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There is an arrangement in the living being, a kind of regulated activity, which must never be neglected, because it is in truth the most striking characteristic of living beings.

—CLAUDE BERNARD,

Lecons sur les Phénomènes de la Vie Commune aux Animaux et aux Végétaux. Vol. 1. Paris, 1879.

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Page 796, table 2, column 4. For m, *read* gm.
Page 798, table 4, column 6. For m *read* gm.
Page 806, table 1, dog HT 8, ♀, 3rd column from right, third line above rule. For 170 *read* 710.
Page 813, 12th line from top of page. For reduction weight, *read* weight reduction.
Page 813, Discussion, 2nd line. For reference (10) *read reference* (2, 10).
Page 814, 11th line above Summary. For reference (11) *read reference* (1).
Page 814, 8th line above Summary. For reference (12), *read reference* (11).
Page 832. For Scholten, Adrian, *read* Scolten, Adrian.

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BIOASSAY OF THE GROWTH HORMONE OF THE ANTERIOR PITUITARY[1]

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BERKELEY, CALIFORNIA

EVIDENCE HAS BEEN ACCUMULATING in favor of the concept that the growth hormone of the anterior lobe of the pituitary is a separate entity, different from the well established pituitary hormones such as the gonadotropic, lactogenic, thyrotropic and adrenocorticotropic hormones (1, 2). Final proof for the individuality of the growth hormone, however, will only be possible when the active principle is obtained in chemically pure form. For the purification of such a biologically active substance, a satisfactory method of assay is necessary, and the efficiency and specificity of the method is of primary importance. There are difficulties, however, in finding a specific and reliable test object for a hormone that controls a phenomenon as complex as somatic growth. The use of gain in body weight of rats for the determination of the growth hormone potency of pituitary extracts was first described by Evans, et al. (3), and is still being used for assay of growth hormone preparations by almost every investigator in this field (4–9).

Freud, Levie and Kroon (10), however, consider growth of the vertebrae of the tail, as determined from skiagrams, as particularly suitable for determination of the growth promoting activity. This reaction is assumed to be more specific and less affected by disturbances of metabolism and disease. However, roentgen measurements are not always easily available, and the relative error is larger in direct tail measurements than in body weight determinations. Attempts to apply other criteria, such as nitrogen retention have not yet proved successful (11, 12, 13). The increase in body weight of rats has so far been used exclusively in this laboratory for routine assay of the pituitary growth hormone. The present paper reports attempts to further standardize this method and to estimate its accuracy.

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[1] Aided by grants from the Research Board of the University of California and from the National Research Council Committee on Research in Endocrinology. Assistance was rendered by the Works Projects Administration—Official Project #65-1-08-62, Unit A-5.

PROCEDURES AND RESULTS

Test Animals

Two types of rats are used in testing for growth hormone. *a*) Normal 'plateaued' female rats, 5 to 6 months of age and weighing from 220 to 280 gm. Although rats of this age have almost reached growth stasis they readily resume growth on administration of growth hormone. *b*) Immature female rats from which the pituitary gland has been removed at approximately 28 to 30 days of age. After hypophysectomy at this age, the body weight continues to increase slowly for a few days only. The animals are therefore not used for the assay until 10 to 12 days after operation. They can be used at later postoperative periods, as the response is similar for at least a month.

When growth hormone injections are given to hypophysectomized rats, beginning immediately after operation, a greater increase in body weight results. But the corresponding untreated controls also grow for a few days, so that the differential growth does not markedly exceed that of rats in which injections are begun after a longer postoperative period. Since rats taken for experimental work immediately after operation are in better general condition and have more pituitary and target organ hormones still in circulation, it may be desirable to use them in some instances.

Hypophysectomized rats are not used for assay, unless evidence for completeness of the operation has been obtained. For this purpose, the following criteria are used. *a*) Limitation of body weight gain to 7 gm. in the first 8 days after operation; *b*) impairment of body tonus; c) maintenance of infantile hair; and *d*) the final head check at autopsy.

Method of injection. The hormone preparations are injected daily in physiological saline in volumes of 0.5 to 1.0 cc. for periods of 10 to 20 days (Sundays excepted). The response was found to be the same, within the limit of error of the method, for intraperitoneal and subcutaneous injections.

Relationship between Dose and Response

In order to establish a basis for subsequent computations of growth hormone potency of unknown fractions, several standardization experiments were performed. The experimental procedure was essentially the same for both normal plateaued and hypophysectomized rats with the exception of the dose levels, which were necessarily higher for normal rats. Five groups of 15 rats each were selected, uniform in regard to age, weight, and, in the case of hypophysectomized rats, postoperative period. The growth hormone preparation used was a 'globulin fraction,' obtained from beef anterior pituitary as described elsewhere (2). Five solutions of decreasing concentration were prepared from a stock solution by successively diluting with an equal volume of saline. The rats were injected daily for 20 days (17 injections) and their body weights recorded every 5 days. A complete diet was given *ad libitum* (*diet XIV* and lettuce; in the case of hypophysectomized rats supplemented by McCollum's *diet I* moistened).[2]

Normal rats. The results of a typical experiment with normal plateaued rats are given in figure 1 which shows the relationship between dose and response. The daily dose is plotted as abscissa, expressed in mg. of dry substance, on a logarithmic scale, the body weight gain as ordinate, expressed in gm. It is obvious, within the limits of dosage used, that a straight line relationship exists between the logarithm of the dose and the body weight gain. This is in agreement with previous findings (1, 8).

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[2] *Diet I*: 67.5% whole wheat (ground); 15% casein; 10% whole milk powder; 1% NaCl; 1.5% calcium carbonate; 5% milk fat (melted butter).

Diet XIV: 67% whole wheat (ground); 5% fish oil; 5% casein; 10% alfalfa leaf meal; 10% fish meal; 3% NaCl.

A comparison of the growth response at succeeding 5-day intervals indicates that the slope of the line increases with increasing periods of injection, up to 15 days. As the increment in weight gain increases with the slope of the curve, the accuracy of the test necessarily increases with the duration of the injections up to a period of 15 days.

In order to determine the accuracy of the method, the statistical significance was determined for the difference in growth response caused by a dose variation of 100%. The body weight increments corresponding to 2 successive dilutions were calculated ($=D$), and standard errors were computed for these increments ($=S_D$) according to Fisher's method for small samples (14). The results are given in table 1. It is obvious

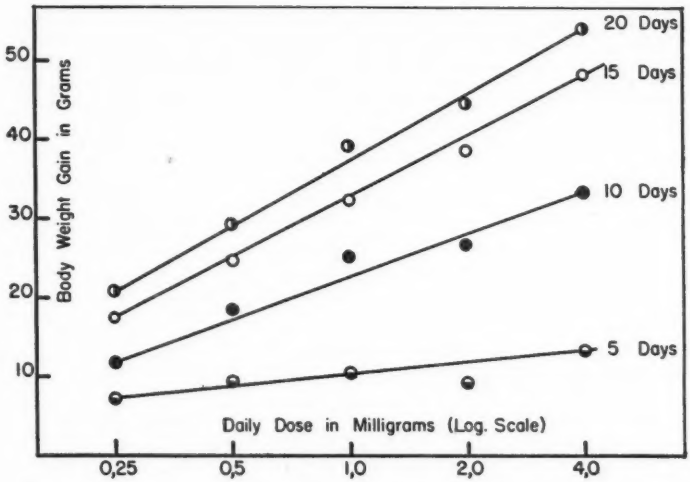

Fig. 1. Response of normal plateaued rats to successive dilutions (1:1) of a growth hormone preparation (Globulin Fraction)

that the larger D/S_D, the more significant are the results. The results were called *significant* when $D/S_D > 2.0$ (at least 95% of cases within limits of S_D); *highly significant* when $D/S_D > 2.75$ (at least 99% of cases within limits of S_D; 2 groups of 15 rats each) (14). The mean values in table 1 indicate that doubling a given dose level causes an average body weight gain increment which is not significant for the 5-day period, but which is significant for the 10 and 20-day periods, and highly significant only for the 15-day period (2 groups of 15 rats each).

The smallest multiples of a given dose level which are necessary to produce a statistically significant change in body weight gain, can be calculated from the mean values for S_D (table 1) and from the diagrams in figure 1. The resulting values (table 2) again indicate that, in this experiment, the highest degree of accuracy was obtained with a 15-day injection period.

Hypophysectomized rats. The results of a typical standardization experiment using hypophysectomized rats are given in figure 2. When the average body weight gain in grams is again plotted as ordinate against the logarithm of the daily dose as abscissa, qualitatively the same diagrams are obtained as with normal animals. Again, a straight line relationship exists within a certain dose range. The highest levels, however, are beyond the dosage range of straight line relationship, where the curve gradually approaches a plateau. This is a general observation for both types of test animal, and is in

TABLE 1. DIFFERENCES IN AVERAGE BODY WEIGHT RESPONSE (D IN GM.) OF GROUPS OF RATS RECEIVING SUCCESSIVE DILUTIONS (1:1) OF A GROWTH HORMONE PREPARATION AND STANDARD ERROR OF THE DIFFERENCE (S_D)

Successive dilutions (daily doses, in mg.)	No. rats	Period of injection in days							
		5		10		15		20	
		D	S_D	D	S_D	D	S_D	D	S_D
Normal female rats									
4.0/2.0	30	4.1	2.4	6.5	2.8	9.6	3.0	9.4	4.9
2.0/1.0	30	−1.4	2.6	1.7	2.3	6.2	2.4	5.5	4.3
1.0/0.5	29	1.2	2.7	6.6	2.2	7.8	2.3	10.0	3.5
0.5/0.25	29	2.2	2.1	6.6	2.1	7.2	2.2	8.3	3.1
Mean values of D and S_D		1.5	2.5	5.4	2.4	7.7	2.5	8.3	4.0
D/S_D		0.6		2.3		3.1		2.1	
Hypophysectomized female rats									
0.48/0.24	26	−0.4	1.8	−2.2	3.5	2.3	3.5	4.9	3.8
0.24/0.12	24	1.9	1.8	4.1	2.4	7.0	3.0	14.0	3.7
0.12/0.06	21	−1.3	1.6	2.1	2.2	4.4	2.9	5.6	3.1
0.06/0.03	21	1.9	1.4	4.2	2.1	6.1	2.8	3.9	3.3
Mean values[1] of D and S_D		0.8	1.6	3.5	2.2	5.8	2.9	7.8	3.4
D/S_D		0.5		1.6		2.0		2.3	

[1] Mean values given only for 4 lower dose levels (0.24 to 0.03 mg.) since highest dose (0.48 mg.) is outside of range of straight line relationship.

agreement with findings of Bülbring and Light, de Beer and Cook (5, 8). Only the straight, steep part of the curve is useful for the assay. Since the slope of the curve increases with the duration of the experiment, the accuracy increases with the injection period, as long as other factors are constant, as in the case of normal rats.

The accuracy of this method for measurement of growth hormone potency in hypophysectomized rats was determined by the same procedure as in normal rats. The body weight gain increments (D) were calculated for successive dilutions, corresponding to dose variations of 100% (table 1). The ratio of the weight increment to the respective standard error (D/S_D) was again used as the index of the degree of significance of the results (table 1). The values corresponding to the dose levels 0.48/0.24 mg. were not included in the computation of the mean values for D and S_D in table 1, since in this range the dose/response curve ceases to be a straight line and begins to reach a plateau. Dose level variations necessary to produce statistically significant

TABLE 2. MINIMAL DOSE LEVEL CHANGES NECESSARY TO PRODUCE STATISTICALLY SIGNIFICANT BODY WEIGHT CHANGES

Test animals		Injection period in days			
Type of rat	No. per dose level	5	10	15	20
		Minimal dose multiples			
Normal	15	11	1.9	1.6	2.0
Hypophysectomized	11	48	2.5	2.1	1.9

body weight changes are again shown in table 2. It can be noted in both tables 1 and 2 that, in this experiment, the 20-day injection period yielded results having the highest degree of accuracy, in spite of great individual variations in response. The 15-day period, however, is only very little inferior in accuracy (table 2).

Number of test animals per group. Another important factor which determines the accuracy of the assay method within certain limits, is the number of test animals used per group. This factor has not been rigorously standardized, since the degree of

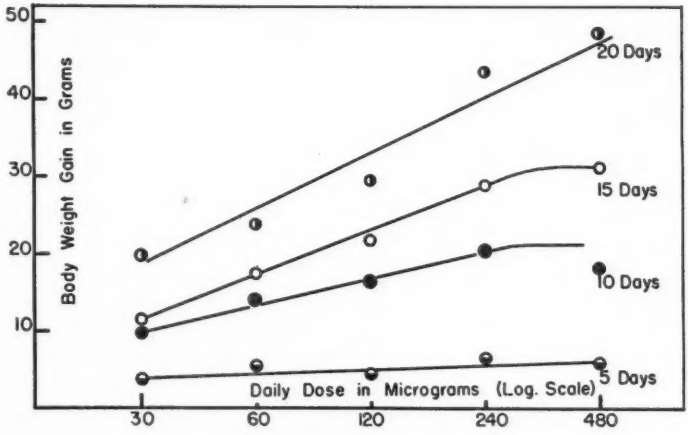

Fig. 2. Response of hypophysectomized rats to successive dilutions (1:1) of a growth hormone preparation (Globulin Fraction).

accuracy required varies in different experiments. In routine work, the number of rats usually ranges from 6 to 12 per group.

Comparison of Normal Plateaued and Hypophysectomized Rats

It has been suggested that hypophysectomized rats give a higher degree of accuracy in the assay (6). It can be noted in tables 1 and 2 that, at present, this is not the case for our rat colony. The accuracy is approximately the same for both types of test animal. It appears to be even somewhat higher for normal rats (table 2) but this is in all probability due to the higher number of animals used in the experiment with normal rats.

There is general agreement, however, about the fact that immature, hypophysectomized rats are more sensitive in their response to growth hormone injections than normal 'plateaued' rats. Table 3 shows results of an experiment in which a group of normal plateaued and one of hypophysectomized rats were injected with the same growth hormone preparation, a 'globulin fraction,' at different dose levels. Since the body weights of these 2 types of test animals were quite different, dose levels and body weight responses were calculated in % of initial body weight. The hypophysectomized rats showed almost a double relative response with only about 1/3 of the relative dose as was given to the normal rats (table 3).

This advantage of higher sensitivity of hypophysectomized rats, however, is offset by the fact that they are in general much more delicate animals than normal rats. Not only is the metabolism of hypophysectomized rats disturbed in many phases, but

they are affected much more than normal rats by the toxicity of some hormone preparations as well as by disease, and by change in temperature.

All of these factors are taken into consideration for choosing the type of animal most suitable for a given case. All preparations available in sufficient amounts are assayed in normal plateaued rats; for more valuable preparations, however, it is preferable to use hypophysectomized rats. Toxic extracts, on the other hand, are assayed in normal rats.

TABLE 3. COMPARISON OF BODY WEIGHT INCREASE OF NORMAL PLATEAUED AND OF HYPOPHYSECTOMIZED RATS INJECTED WITH THE SAME GROWTH HORMONE PREPARATION[1] FOR 10 DAYS

No., type of test animal	Mean body wt. at onset	Daily dose		Body wt. gain per day	
	gm.	*mg.*	*% of body wt.*	*gm.*	*%*
55 Normal	246	1.00	0.41×10^{-3}	2.9	1.2
13 Hypophysectomized	70	0.10	0.14×10^{-3}	1.5	2.1

[1] Globulin fraction.

Growth Hormone Units

Growth hormone units were introduced for both normal plateaued (7) and hypophysectomized (4) rats. The 'normal rat unit' was defined previously for an injection period of 20 days exclusively, the 'hypophysectomized rat unit' only for 10 days. On

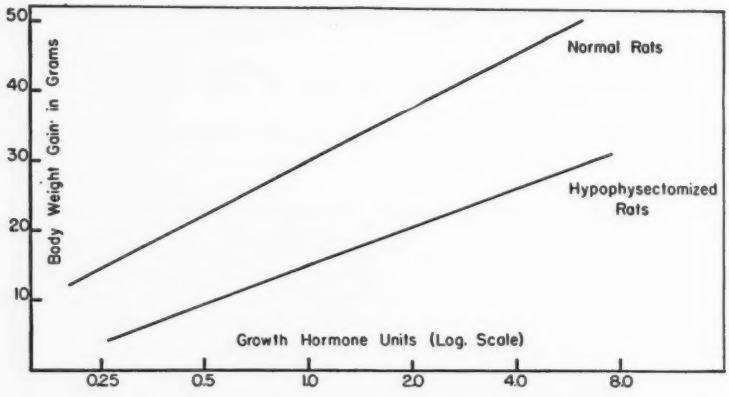

Fig. 3. STANDARDIZATION CURVES, BASED ON UNITS, FOR COMPUTATION OF GROWTH HORMONE POTENCY (15 Day Test).

account of the results of the standardization experiments given in tables 1 and 2 it was decided recently in this laboratory to use also injection periods of 15 days for both types of test animals. A 'normal rat unit' is now defined more generally as the amount of growth hormone that produces, when given daily intraperitoneally for a period of days as specified, an average body weight gain of 2 gm. per day. A 'hypophysectomized rat unit' is defined as equivalent to the amount of hormone that produces an average body weight gain of one gram per day, when given intraperitoneally daily for a period of days as specified. In this way, the definitions are applicable for different

injection periods. Practical considerations which usually result in a compromise between the accuracy desired and the time period involved, determine the duration of injections chosen in a given case.

For the computation of unitage of unknown preparations, diagrams representing the relation dose to response have to be used. The computation is markedly facilitated, and the diagrams acquire a more general character when the dose levels are expressed directly in growth units, instead of in milligrams of a particular preparation. Therefore, curves were constructed with such dimensions for both types of test animals and for all injection periods for which growth hormone units were established. Figure 3

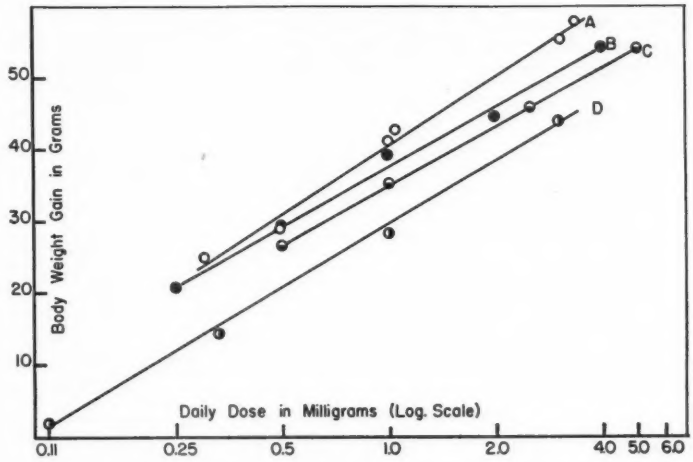

Fig. 4. Response of normal plateaued rats to various dilutions of different growth hormone preparations (20 Day Test).

shows such curves for the 15-day period, for both normal and hypophysectomized rats, constructed from figures 1 and 2 respectively.

Formerly, unknown fractions were injected in 2 or more dose levels, in order to verify in each instance the slope of the dose/response curve. But it was observed that the slopes were in most cases approximately parallel within the limits of error of the method, even with preparations having different potencies, and in spite of variations in the sensitivity of the rats. This is illustrated in figure 4 which shows results of standardization experiments with normal plateaued rats. These have been carried out by different investigators of this laboratory over a period of almost 10 years, using various preparations of different potency. *Curve A* was obtained with an 'L fraction' (7), *B* with a 'globulin fraction' (2), *C* with a crude $Ca(OH)_2$ extract (1) and *D* with a crude $Ba(OH)_2$ extract (3). It is obvious that the slope of the curves remained constant within the limit of error of the method. When curves are constructed for the same experiments with the abscissae expressed in growth units instead of mg. as discussed above, the curves given in figure 4 practically coincide. For these reasons, only one dose level need be given in routine assays. It is realized, however, that the slope of the curve should be checked occasionally, especially when a different chemical procedure is employed in preparation of the hormone.

Effect of Age and Initial Body Weight of Test Animals

Some investigators propose to administer the hormone in doses proportional to body weight of the test animals, and to express the results not as absolute gain but in % of total body weight (6, 8, 9). If the body weight of the test animals varies considerably, such methods of application and computation might be valuable. If it is well standardized, however, there is no advantage in relating dose levels and results to the initial body weight. It has been considered essential in this laboratory for years that the test animals . . . 'be rigorously standardized as to strain, sex, age, weight, physiological condition, etc. . . . ' (7). And it was found that the accuracy of the results was not increased when the data given in figures 1 and 2 were expressed in % of initial body weight.

How important it is to standardize the animals as well as possible, not only as to weight, but also as to their age, is demonstrated by the results of 2 experiments shown in table 4. In each of these experiments 2 groups of hypophysectomized rats of approximately the same postoperative periods, but selected from different age groups to yield animals of different weights, were injected with the same growth hormone preparation in doses proportional to their average body weights. It will be noted that the older animals were relatively less responsive; their body weight gain expressed in % of body weight was smaller than that of the younger and smaller rats. When both dose level and body weight gain were expressed in absolute units, the body weight gains were practically the same for heavier and lighter rats, within the limits of error of the method, while the dose levels varied about 100 and 80% respectively. Thus, it is not possible, to use hypophysectomized rats of different weights and ages in a given assay.

TABLE 4. AVERAGE BODY WEIGHT RESPONSES OF 2 GROUPS OF HYPOPHYSECTOMIZED RATS[1] HAVING DIFFERENT WEIGHTS AND AGES[2] TO A GROWTH HORMONE PREPARATION GIVEN IN DOSES PROPORTIONAL TO BODY WEIGHT

Experiment	Age at operation	Body wt. at onset of experiment	Daily dose		Body wt. gain in 20 days	
	days	*gm.*	*mg.*	*% of body wt.*	*gm.*	*%*
I	27–28	73	0.10	1.4×10^{-4}	29	40
	58–64	141	0.19	1.4×10^{-4}	34	24
II	29–30	70	0.09	1.5×10^{-4}	27	34
	62–65	143	0.16	1.5×10^{-4}	22	16

[1] 10–12 rats per group.
[2] Postoperative period at onset of injections. *Experiment I*: 26–29 days; *Experiment II*: 20–23 days.

Repeated Use of Test Animals for Assays

Various investigators have made the observation that the body weight response of animals to daily injections of a given hormone preparation decreases with increasing duration of the experiment (5, 6, 7). Even in normal rats which, upon continuous administration of growth hormone, continue to grow well into senility, the very rapid growth rate of the first few weeks is never equaled in later periods of treatment. This phenomenon is apparent even when the animals are given, after a preceding course of injections, a rest period of one or a few weeks. Therefore, it is not advisable to use test animals more than once for an assay, when results of the highest accuracy possible are required. However, animals which have been used for a previous assay, still re-

spond readily to growth hormone injections, and can, after a rest period to allow stabilization of weight, be used for preliminary assays in much of the routine work, as long as their limitations are realized. Their response is smaller and more variable, and they cannot be considered the standaridized and dependable test object that is represented by unused rats. It is essential, of course, to use only one type of test animal for a given assay.

Limitations of the Assay Method

The responsiveness of the rats varies considerably at different times of year. Therefore, a standard reference substance should be used, when absolute values for the potency are desired (1, 8). The standard substance is kept as dry powder in vacuo over P_2O_5 at approximately $2-5°$ C. Under such conditions no loss in potency became evident during a period of 9 months. However, the possibility of a slow deterioration even under such conditions has to be taken into consideration for longer periods (6).

The body weight gain resulting from injection of a given pituitary preparation is determined not only by the concentration of the growth hormone present in the extract. Two phenomena in particular have to be mentioned which may have a considerable influence on the body weight response. *a*) The rate of absorption of the hormone, b) synergic and antagonistic effects of other pituitary hormones present in growth hormone preparations.

It has been recognized in the case of other hormones such as insulin (15), and gonadotropins (16) that the rate of absorption is of primary importance for the response. Contaminating substances present in growth hormone preparations, such as inert proteins or inorganic salts, especially in earlier stages of purification, may form less soluble complexes with the hormone and, therefore, may have a decided influence on the rate of absorption in the body and, in consequence, on the apparent response.

The other phenomenon, synergism and antagonism of other pituitary hormones present in an extract, also may have a decided influence on the observed body weight gain. A synergistic effect of thyrotropic hormone at relatively low levels, as well as, at least in normal rats, an inhibiting action of high doses of adrenocorticotropic hormone may be expected (17, 18). A study of the effects on body weight gain of various highly purified hormones of pituitary origin combined with purified growth hormone will be the subject of a separate publication.

It is evident that both of these phenomena impose difficulties in the interpretation of the results of bioassays of preparations of different degrees of purity. It is easily conceivable that through fractionation synergistic factors are removed from a growth hormone preparation, so that the final product, though actually containing the growth hormone in higher concentration and in a higher state of purity, appears not increased, or even decreased, in its growth hormone potency. It is not sufficient therefore, in tracing purification of the growth hormone, to follow the change in growth hormone potency alone. It is desirable to follow also the content of other pituitary hormones which contaminate the preparations, with specific assay methods.

SUMMARY

A method was described for the quantitative determination of the pituitary growth hormone in both normal 'plateaued' and hypophysectomized immature rats.

The relation between the logarithm of the daily dose and the body weight gain could be represented by a straight line within a limited range of doses. The slope of this line increased with the duration of injections, when different periods of injection

were compared. Curves representing the relationship dose to response for different growth hormone preparations, had a constant slope, even if the growth hormone potency was different, when the period of injection was kept constant.

Growth hormone units were defined for each type of test animal. Curves representing the relationship dose to response were given in which the abscissa (logarithm of dose level) was expressed in growth units, in order to facilitate computation of unitage.

In order to estimate the accuracy of the method, body weight increments were calculated resulting from doubling a given dose level, and corresponding standard errors were computed. Minimal dose level changes necessary to produce statistically significant body weight changes were thus determined, also injection periods giving the highest degree of accuracy.

The 2 types of test animal were compared as to their suitability and limitations of the method were described. Seasonal variations in responsiveness of the rats were discussed and the importance of a standard reference substance was stressed.

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GONADOTROPIC HORMONE IN AP OF MALE AND FEMALE RABBITS DURING GROWTH[1,2]

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FOR A NUMBER OF YEARS THIS laboratory has been engaged in a systematic study of the changes in the concentration of the various hormones in the pituitary of experimental animals during growth, pregnancy and lactation. It was hoped that these studies would contribute *a*) to the question of the relation between the concentration of the various hormones in the pituitary and the rate of secretory activity, *b*) to the problem of the relation of the concentration of the AP hormones and the sensitivity of the glands or organs stimulated, *c*) to the species and sex variations under comparable conditions, and finally *d*) to the interrelation of the hormones during growth, attainment of sexual maturity, pregnancy, lactation, growth stasis, and fattening.

In preceding reports, the changes in the lactogenic (1) and thyrotropic (2) hormones of the AP of New Zealand white rabbits during growth have been described. In the present report similar data are presented concerning the changes in the gonadotropic hormone of the pituitary in the groups of rabbits used for study of the thyrotropic hormone.

EXPERIMENTAL PROCEDURE

The experimental procedure concerning the feed and care of the rabbits, their grouping as to weight, the preparation of the pituitaries for assay, and other related data is reported in an earlier paper (2). Further, the male baby-chicks used for the assay of the thyrotropic hormone were used at the same time for the assay of the gonadotropic hormone. This was made possible by the close relation between the concentration of thyrotropin and gonadotropin in the rabbit pituitary required for a unit response.

In a previous paper (3) we defined a chick unit of gonadotropic hormone as the total amount of hormone administered over a 4-day period with subcutaneous injections once each day, which would elicit a mean weight increase of 50% (to about 13.2 mg.) in the gonads of 20 male chicks weighing an average of 55 ± 10 gm. The normal gonad weight was reported as about 8.8 mg. at that time.

Control data on the normal weight of the chick gonads (White Leghorn cockerals) during a period of 4 years is presented to show certain variations which have occurred during this period (table 1). During the early part of 1939 when the animal room was being reconstructed we were in temporary quarters where the heat control was unsatisfactory. To warm the chicks, an electric light was placed over them and kept burning continually during the 4-day injection period. Under these conditions the

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TABLE 1. GONAD WEIGHT OF NORMAL MALE BABY CHICKS

Year	No. of chicks	Body wt. av.	Gonad wt. av.	Standard error of mean	Room temperature
		gm.	mg.		° F.
1937	20	52.0	8.7	±0.56	70–90
1938	20	55.3	9.0	±0.51	70–90
1939	48	56.3	13.2	±0.55	55–85. Additional heat supplied with 100 watt light bulb
1939	49	56.9	11.2	±0.58	55–85. No extra heat supplied
1939	37	55.2	8.7	±0.39	78–82.[1] No extra heat supplied to cage
1939	107	52.3	8.9	±0.28	78–82.[1] Extra heat supplied by electric brooder, thermostatically regulated
1940	127	52.2	9.1	±0.26	Same
1941[2]	88	53.6	9.8	±0.30	Same

[1] Thermostatically controlled room temperature. The lights in the animal room are kept burning from 6 A.M. to approximately 7 P.M.

[2] To Aug. 9.

gonad weight of the controls increased markedly and a group of 48 chicks had gonads which averaged 13.21 mg. When the lights were eliminated but the temperature was still quite variable, the gonads of the normal chicks remained distinctly above the previous normal level. Small electric brooders (thermostatically regulated) were placed in the cages which furnished additional heat to the chicks in the thermostatically controlled animal room. The temperature of the brooder was approximately 95° F. The lights in the animal room were kept burning from 6 A.M. to approximately 7 P.M. Under these conditions it will be noted that the normal gonads returned to the previous weight level. Our experiences are related because it appears important that temperature and light be carefully controlled if the chick is to be used for assay purposes, especially of the gonadotropic or androgenic hormones (4).

TABLE 2. GONADOTROPIC HORMONE CONTENT OF GROWING RABBIT PITUITARIES

No. of rabbits in group	Rabbit		Amt. pit. injected /chick, mg.	No. of chicks in group	Wt. of chicks av., gm.	Chick gonads wt. av., mg.	Chick unit content			Gonad wt. of rabbits av., mg.
	body wt. av., gm.	pit. wt. av., mg.					Per gland	Per gm. of pit.	Per 100 gm. body wt.	
					Males					
19	149	4.7	24	3	51.0	8.8	—	—	—	—
43	517	10.6	20	20	53.8	12.4	0.387	36.5	0.075	86
28	995	13.2	16	20	53.1	15.1	1.084	82.1	0.191	242
18	1522	21.3	14	20	54.6	15.9	2.266	93.1	0.149	599
15	2009	21.2	13	20	56.5	19.1	3.612	165.3	0.180	2009
11	2503	26.3	13	20	54.1	21.0	5.298	201.5	0.212	4061
13	3003	27.0	13	20	53.9	18.7	4.454	164.8	0.148	5768
10	3483	33.3	14	20	52.4	19.5	5.454	163.6	0.157	6757
4	4128	28.7	15	7	52.3	19.0	4.145	144.5	0.100	9697
					Females					
18	163	4.5	24	3	54.0	8.1	—	—	—	—
43	517	10.6	20	20	54.1	14.3	0.610	57.2	0.118	19
25	1013	16.3	16	20	54.3	13.8	1.006	61.5	0.099	41
17	1501	23.4	14	20	54.3	16.0	2.459	105.2	0.164	138
13	2032	23.6	13	20	54.0	15.2	2.430	102.8	0.115	160
11	2489	30.4	13	20	54.2	15.3	3.123	105.4	0.125	228
10	3054	31.2	13	20	54.5	14.2	2.713	86.9	0.089	275
8	3544	45.0	14	20	53.5	15.2	4.296	95.3	0.121	602
8	4021	41.4	15	20	53.8	15.6	3.973	96.0	0.099	833
8	4561	40.2	15	20	52.1	10.9	1.054	26.2	0.023	816

It is also important to continually sample the chicks to obtain normal gonad weights as a check on the experimental weights. During the year 1940 in which the assays to be reported in this paper were conducted, the average normal gonad weight was 9.10 mg. when the chicks were killed on the 5th day. The unit response is considered to be a 50% increase in gonad weight above that average. It would appear from the study of Breneman (5) that the weight increase in the male chick gonad is due primarily to the FSH content of the pituitary, although LH appeared to have some

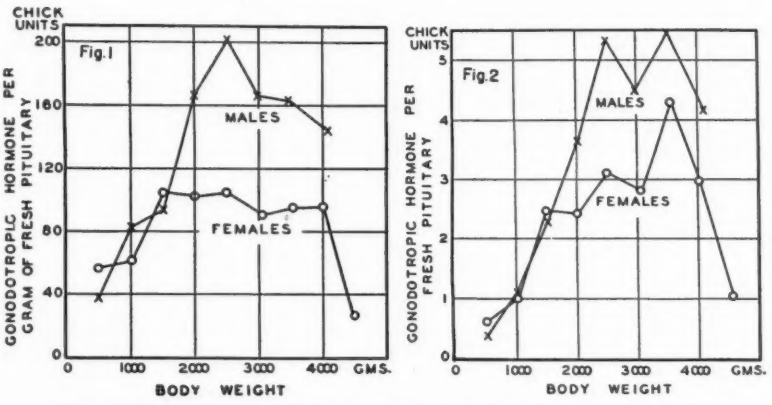

Fig. 1. WITH INCREASING BODY WEIGHT IN NEW ZEALAND WHITE RABBITS, the concentration of the gonadotropic hormone increases for a time, then plateaus until old age. The males show a longer period of use and maintain a higher level of concentration than do the females. Fig. 2. WITH INCREASING BODY AND PITUITARY WEIGHT, the average amount of gonadotropic hormone per pituitary gradually rises in both sexes.

effect. There is also the problem of synergism of the two factors in the unfractionated AP which has not been studied in the chick.

OBSERVATIONS

In male rabbits, the gonadotropic hormone per gm. of fresh pituitary tissue was low in the group weighing 500 gm. or less (table 2). In the group weighing 1,000 gm. the concentration had more than doubled and continued to rise steadily until a body weight of about 2,500 gm. was attained. The gonadotropic hormone concentration then receded slightly and became stabilized at about 160 chick units per gm. of fresh pituitary from rabbits of increased body weight (fig. 1). The hormone content per pituitary and per 100 gm. of body weight followed essentially the same trend (fig. 2).

The testis weight in the groups weighing 500 and 1,000 gm. remained low in association with the low gonadotropic hormone content. The testes increased in weight as the hormone concentration increased. However, the testes continued to increase in weight throughout all body weight classes (fig. 3) even though the gonadotropic hormone concentration remained contant in the pituitaries of animals weighing above 3000 gm. .

The concentration of gonadotropic hormone in the pituitary glands of the females was somewhat higher in the group weighing 500 gm. than in the corresponding class of males. The content was essentially unchanged in the animals weighing 1000 gm. but in the group weighing 1500 gm. there was a rise of about 100% in concentration with a plateau at approximately this level throughout the reproductive

life of the animals. In the heaviest group of animals which were very fat, the concentration of gonadotropic hormone was reduced to about the prepuberty level. It would thus appear that the female rabbit may reach a low stage of gonadotropic function in old age.

The average ovarian weight of the groups of animals weighing up to the 3,000 gm. increased slowly, with an acceleration in the group weighing 1,500 gm. These were virgin animals and presumably without previous or current corpora lutea. The groups

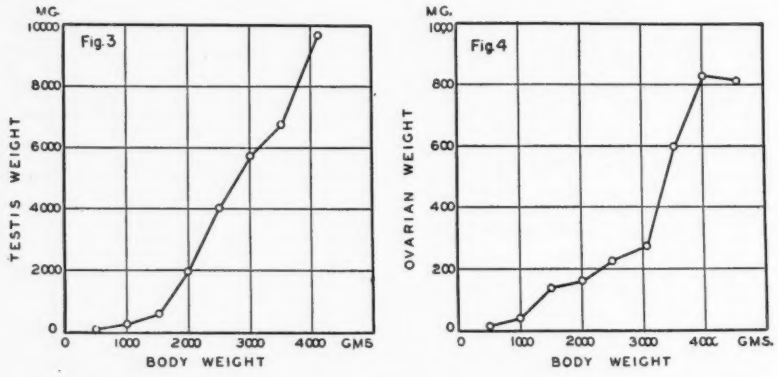

Fig. 3. THE TESTIS WEIGHT OF NEW ZEALAND WHITE RABBITS GRADUALLY INCREASES WITH INCREASING BODY WEIGHT. However, the rise is very slow until about 2,000 gm. weight is attained. Fig. 4. RELATION OF BODY WEIGHT TO OVARIAN WEIGHT OF NEW ZEALAND WHITE RABBITS. The groups up to and including 3,000 gm. were virgin does whereas the heavier groups were multiparous animals. The presence of corpora lutea in various stages of regression appears to increase the ovarian weight considerably. The pituitary weights of this group were also greater than the virgin groups.

weighing more than 3,000 gm. were multiparous animals and showed ovarian weights considerably above those of the lighter weight groups (fig. 4).

It is interesting to note also that the pituitary weights of this group of multiparous animals were considerably above those of the groups of virgins.

DISCUSSION

The data presented in this paper are believed to contribute to the question of the relation of the gonadotropic hormone concentration in the AP and the rate of secretion of the hormone. The rise in concentration of the gonadotropic hormone with body weight and age is related to the gradual increase in gonad weight. With sexual maturity the pituitary of the female rabbit appears to secrete a rather uniform amount of gonadotropic hormone in the absence of pregnancy or pseudo-pregnancy. These observations are as might be expected considering the fact that rabbits remain in estrum for long periods unless interrupted by pregnancy or pseudo-pregnancy. In such animals there would develop a state of equilibrium between the rate of secretion of the gonadotropic hormone by the pituitary and the secretion of estrogen by the ovaries.

Some evidence was obtained for the development of a reduced gonadotropic hormone secretion rate in very heavy and presumably old female rabbits which might be considered to be comparable to conditions in the climacteric. These animals were obtained from a breeder because they failed to breed.

The gonadotropic hormone in the AP of the male rabbit reached a maximum at a slightly later stage of body growth than the female and maintained a level of concentration per gm. of pituitary of about 70% more than the female.

SUMMARY

A study is reported concerning the amount of gonadotropic hormone in the pituitaries of groups of male and female New Zealand White Rabbits of increasing body weight. This was measured by the gonad weight increase of baby chicks. On the basis of the chick units of hormone per gm. of fresh pituitary, it was observed that there was a gradual rise in hormone concentration reaching a maximum in the group of females weighing 1,500 gm. and in the males weighing between 2,000 and 2,500 gm. The female AP especially showed a rather uniform hormone concentration throughout reproductive life except for a notable decrease in the heaviest weight class. This decrease is interpreted as an indication of the climacteric. The gonadotropic hormone concentration of the AP of sexually mature males averaged about 70% above that of females of comparable weight.

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THE GONAD-STIMULATING POTENCY OF THE PITUITARY OF HYPOTHYROID YOUNG MALE RATS

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INTRODUCTION

THE EFFECT OF THYROIDECTOMY on the histological picture of the pituitary in mammals has been noted by various investigators. All agree that the eosinophiles become degranulated and decrease in number and that there is increased vacuolation in the cells of the gland. There have been differences of interpretation concerning the significance of the vacuolation, especially of the basophiles. Whether this change in the histological picture of the pituitary signifies a change in the gonad-stimulating potency has been a contested point. Evans and Simpson (1) and Van Dyke and Ch'En (2) have produced evidence indicating impairment of the ovarian-stimulating function of the pituitary following thyroidectomy. On the other hand Smith and Engle (3) Van Horn (4) and Smelser (5) have reported little if any change in gonad-stimulating properties due to the operation. The present experiments in which young male rats were thyroidectomized and their pituitaries tested for gonad-stimulating potency, were undertaken with the hope that the results might help in the solution of this disputed problem. The first series of experiments was begun in 1935, the second in 1938. Since the completion of the work several reports of similar experiments have come to the attention of the authors. Reforzo Membrives (6) and P'An (7) reported a decrease in the gonad-stimulating potency after thyroidectomy in rats and rabbits. Okano and Tanaka (8) in their experiments with thyroidectomized rabbits found that the gonadotropic potency was increased after the operation.

PROCEDURE

Sixty-four young male rats constituted the donors for the first experiment and 6 were used for a histological study of the pituitary. Of these two groups 32 and 3, respectively, were thyroidectomized at 21 to 28 days of age and their littermates served as controls. After a period ranging from 21 to 48 days following thyroidectomy the pituitaries were injected through a capillary pipette into the leg muscle of immature female mice 20 to 24 days of age. One littermate served as an uninjected control (*C series*), one received 1 or 2 pituitary glands from normal donors (*P series*), and a 3rd was implanted with pituitary from 1 or 2 thyroidectomized donors (*T series*). Donor body weights were taken at the time of thyroidectomy and again when the animals were killed; the donor pituitaries were weighed before injection. For 13 sets of hosts the individual dosage was 2 glands, given one at a time 2 days apart; 6 received only 1 pituitary per host. All hosts were killed at the end of the 4th day.

The second series of experiments was performed a year later using 14 control and 17 experimental donors which were thyroidectomized at 21 to 23 days of age and killed 50 days after operation. The donor pituitaries for this series were desiccated

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with acetone, extracted with pyridine, and injected in distilled water solution. Each host received either 2 or 3 glands administered in 5 daily injections and hosts were killed at the end of the 5th day.

The operated donors were examined macroscopically for thyroid tissue and in the majority of cases microscopic examination was made of the throat region. Six sets of hosts were eliminated because of the presence of a considerable remnant of thyroid tissue in one or more of their donors; in 2 of these donors the thyroid remnant could be seen macroscopically. No data from these animals are included in this paper.

In all host animals the patency of the vagina and stage of the estrous cycle at the time of death were noted. Ovaries and uterine tubes were weighed and fixed in Bouin's fluid, cut at 10μ and stained in Delafield's hematoxylin and eosin. Body weights of hosts were recorded at the time of the first injection and again before death.

The pituitaries of 3 thyroidectomized animals and their littermate controls were sectioned serially at 5μ and stained in Mallory's connective tissue stain.

A histological study was made of 6 sets of the host ovaries and the numbers of various types of follicles counted. The count was checked and rechecked by an assistant who did not know the history of the ovaries. Following the method of Lane no follicle with less than 2 layers of cells was counted and a follicle was considered vesicular if there was obvious separation of the layers of cells from each other. If the antrum was comparatively large and the cumulus oöphorus attached in only one region to the outer granulosa layers a large vesicular follicle was recorded (table 3). Atretic follicles were classified as primary or vesicular on a similar basis. Small vesicular follicles had several layers of atretic cells with irregular spaces between them; large vesicular follicles had a continuous antrum around the egg. The eggs in the small atretic vesicular follicles were degenerate and often fragmented; pseudomaturation spindles were fairly frequent in those of the large atretic vesicular variety. Primary atretic follicles were not listed.

OBSERVATIONS AND RESULTS

Effect of thyroidectomy on the weight and histology of the pituitary. The removal of the thyroid resulted in an increase in the weight of the pituitary in relation to body weight in 17 of the 20 cases in *series 1*. There was an actual average increase of 0.6 mg. and an average increase relative to body weight of 0.0008%. In *series 2* the average increase was 2.08 mg. and 0.0025% relative to body weight.

Histologically the pituitaries of the thyroidectomized animals showed a decrease in the number of eosinophiles and a corresponding relative increase in number of chromophobes and basophiles. The eosinophiles decreased in size as well as number. Vacuolation was pronounced and characterized the basophiles and chromophobes chiefly.

Effect on gonad-stimulating potency. The data obtained in *series 1* on the weight of the tubes and ovaries of *a*) control mice, *b*) mice implanted with pituitaries of normal donor rats and *c*) those receiving glands of thyroidectomized donors were examined. First analysis showed no consistent difference between the two latter groups and the average difference was too small to be convincing. A brief report of the work was made at this point (10). Microscopic examination of sections of the throat regions of the donor rats later revealed the presence of varying amounts of thyroid tissue in some of the operated animals. In 4 of the supposedly thyroidectomized donors there were active thyroid follicles in more than 150 sections of the throat region. The hosts to these donors showed increased ovarian and tube weights over those of hosts to pituitaries from unoperated control animals. These cases have been omitted entirely. In table 2 the data on the remaining hosts and their controls in *series 1* have been aver-

TABLE 1. PITUITARY AND BODY WEIGHTS OF NORMAL AND THYROIDECTOMIZED DONOR MALE RATS

Series	No. of host litter	Pituitary weight in mg.		Body weight in gm.		$\frac{\text{Pituitary weight}}{\text{Body weight}} \times 100$	
		Normal	Thydect.	Normal	Thydect.	Normal	Thydect.
1	2	6.4	9.6	161	195	.0039	.0049
		6.4	5.2	157	134	.0041	.0039
	3	6.0	5.8	157	131	.0038	.0044
		7.4	5.8	134	168	.0055	.0035
	6	4.4	5.0	121	128	.0036	.0039
		4.0	4.6	121	123	.0033	.0037
	7	4.2	4.6	110	102	.0038	.0045
		3.8	4.0	111	103	.0034	.0037
	8	3.0	4.2	137	80	.0022	.0053
		4.2	3.4	90	94	.0047	.0036
	9	3.9	7.4	120	126	.0033	.0050
		5.8	7.8	157	135	.0037	.0058
	16	6.4	4.6	166	95	.0039	.0048
		5.6	4.8	166	102	.0034	.0047
	18	6.8	7.8	201	193	.0034	.0041
	19	6.8	7.0	174	154	.0039	.0046
	20	5.2	6.0	159	134	.0033	.0045
	21	4.4	6.6	147	133	.0030	.0050
	24	5.8	7.0	143	133	.0041	.0053
	25	5.4	6.8	126	116	.0045	.0058
	Av.	5.3	5.9			.0037	.0046
2	1a	5.1	9.9	178	198	.0029	.0050
		5.1	9.8	184	202	.0028	.0049
	1b	6.1	9.2	175	177	.0035	.0052
		6.5	9.4	188	158	.0035	.0059
	2a	5.1	5.9	149	135	.0034	.0066
		6.7	9.1	181	123	.0037	.0057
		6.9	6.9	171	130	.0040	.0067
	2b		8.9		109		.0050
			7.0		112		.0081
			8.7		115		.0060
	3	7.8	9.6	206	139	.0038	.0069
		5.5	7.4	168	132	.0033	.0056
		5.3	8.6	159	116	.0033	.0074
	4	5.6	5.6	137	113	.0041	.0050
		5.7	6.5	133	113	.0043	.0058
	5	7.0	7.8	180	125	.0039	.0062
		6.6	8.5	155	124	.0043	.0069
	Av.	6.08	8.16			.0036	.0061

aged. In the *T* as compared with the *P* series there was an average decrease in ovary weight of 0.8 mg. and in tube weight of 5.1 mg. These are slight differences. That of the ovaries is approximately twice its probable error while that of the uterine tubes is 2.2 times its probable error. The condition of the vagina is also recorded for each host in table 2, *series* 1. Vaginal opening had not occurred in 5 cases in the *T* series as compared with only 2 cases in the *P* series.

The data from *series* 2, in which the pyridine-extracted pituitaries were injected over a longer period of time, are more convincing (table 2). Ovaries of hosts injected with pituitaries of thyroidectomized donors (*T*) weighed 50% less relative to body weights, 68% less in actual weights, than those of hosts to pituitaries from normal donors (*P*). The increase in ovarian weights over those found for unoperated control mice was 83% for the *P* hosts, 11% for the *T* hosts. The average weights of the uterine tubes were 14.6, 28.6 and 17.5 mg. for the control, the *P* and the *T* series

respectively. The average differences between the *P* and *T* hosts are significant for both ovaries and tubes, being approximately 5 times the probable error for the former and 3½ times for the latter. The vaginal opening had occurred in 4 of 5 cases for the *P* hosts while the ratio for the 7 *T* hosts was 2 open to 5 closed.

Histological examination of ovaries. There was some individual variation in the number of follicles of various types as recorded for the 6 sets of ovaries in table 3. The total number of follicles showed an increase of 31.3% in the *P* series and of

TABLE 2. DATA FOR CONTROL MICE (C), HOSTS TO NORMAL DONOR PITUITARIES (P) AND TO PITUITARIES OF THYROIDECTOMIZED DONORS (T)

Series	No. of host litter	Wt. of ovaries, mg.			Wt. of tubes, mg.			Vagina		
		C	P	T	C	P	T	C	P	T
1	2	1.8	3.6	3.6	9.8	19.4	13.2	—	—	—
	3	2.0	3.8	3.4	19.2	24.0	22.0	closed	open	open
	6	2.2	3.0	2.6	9.2	32.2	38.0	closed	open	open
	7	—	3.4	1.6	—	22.4	14.8	—	open	closed
	8	3.0	6.8	7.4	6.6	30.0	24.0	closed	open	open
	9	1.9	6.0	4.6	7.0	49.8	18.6	closed	open	open
	16	3.2	11.6	5.0	13.6	40.2	40.4	closed	open	open
	18	2.2	3.4	2.6	13.0	15.0	14.4	closed	open	closed
	19	3.0	4.2	3.2	10.2	23.8	13.8	closed	open	closed
	20	3.2	3.4	4.6	11.6	21.0	19.6	closed	open	closed
	21	2.4	4.2	3.6	12.6	26.4	19.4	closed	closed	open
	24	2.0	2.0	2.0	12.0	15.2	18.4	closed	closed	open
	25	1.6	1.6	2.4	9.4	19.4	16.2	closed	open	closed
	Av.	2.4	4.4	3.6	10.4	26.1	21.0			
2	1a	1.7	—	2.2	14.0	—	16.8	closed	—	closed
	1b	—	2.9	2.3	—	36.0	14.0	—	open	closed
	2a	2.0	4.2	2.1	9.0	21.0	13.8	closed	closed	closed
	2b	—	—	2.1	—	—	24.0	—	—	open
	3	1.8	2.8	2.6	17.1	22.8	18.2	closed	open	open
	4	2.7	5.6	2.8	21.8	45.2	19.8	closed	open	closed
	5	2.1	3.6	1.7	11.0	18.0	16.2	closed	open	closed
	Av.	2.1	3.8	2.3	14.6	28.6	17.5			

16.3% in the *T* series over the average number in control animals. The average numbers of small vesicular follicles, of large vesicular follicles and of luteinized follicles were greater in the *P* than in the *T* series. When the vesicular and luteinized follicles are considered together as a percentage of the total number the same relationship exists. This is true within each litter as well as in the average for all the litters. In every case the *P* host showed a greater percentage of vesicular plus luteinized follicles than the *T* host. The difference is not large, an average of 10% with a range of 5.1% to 13.9% but is as great as the average difference between the control animals (*C series*) and the *T* series.

Counts of follicles were not made for the second series of experiments but observation of the ovaries gave evidence that there were more and larger vesicular follicles in the hosts to normal donor pituitaries than in those which received glands from thyroidectomized donors.

DISCUSSION

Changes in the weight and histological appearance of the anterior lobe of the pituitary after thyroidectomy have been noted by numerous investigators (11). The

pituitary weight increased in rabbits (12, 13), sheep (14) and male rats (6). Hammett (15, 16) found a consistent pituitary hypertrophy in the male but not in the female rat and Schooley (17) reported no increase in thyroidectomized pigeons. In general the longer the period of thyroid deficiency the greater was the hypertrophy. Increase of pituitary weight was noted in this study of hypothyroid rats and it characterized the absolute weights in most cases as well as the weights of the glands relative to body weights.

The histological picture, however, seems to indicate a decreased rather than an increased functioning of the pituitary gland. Reduction in the size, number and granular content of the eosinophiles was observed in this study of hypothyroid young male

TABLE 1. COMPARATIVE NUMBERS OF FOLLICLES OF VARIOUS TYPES IN OVARIES OF CONTROL MICE (C), HOSTS TO NORMAL DONOR PITUITARIES (P) AND TO PITUITARIES OF THYROIDECTOMIZED DONORS (T)

Litter no.	Total no. of follicles			Primary follicles			Advanced follicles			Subdivisions of advanced follicles											
										Vesicular Small			Vesicular Large			Lutein-ized			Atretic small			Vesicular large		
	C	P	T	C	P	T	C	P	T	C	P	T	C	P	T	C	P	T	C	P	T	C	P	T
9*	262	370	277	128 48.9%	98 26.5%	102 36.8%	134 51.1%	272 73.5%	175 63.2%	100	166	93	2	20	1	0	42	44	6	40	18	26	4	19
16	159	338	179	76 47.8%	77 22.8%	65 36.3%	83 52.2%	261 77.2%	114 63.7%	61	135	60	0	24	24	0	56	3	2	16	13	20	30	14
19*	170	201	174	68 40%	28 13.9%	33 19%	102 60%	173 86.1%	141 81%	62	87	49	0	22	9	0	19	8	12	23	21	28	22	54
21	—	217	207	—	50 23%	73 35.3%	—	167 77%	134 64.7%	—	73	69	—	12	2	—	2	0	—	20	16	—	60	47
24	—	190	207	—	53 27.9%	72 34.8%	—	137 72.1%	135 65.2%	—	77	55	—	0	0	—	0	0	—	42	33	—	18	47
25	139	122	229	50 36%	31 25.4%	90 39.3%	89 64%	91 74.6%	139 60.7%	63	44	85	1	1	1	0	0	0	6	21	9	19	22	41
Av.	183	240	212	80.5 43.2%	56.2 23.2%	72.5 33.6%	102 56.8%	183.5 76.8%	139.7 66.4%															

* Counts for C animals are those from one ovary doubled.

rats. Similar changes have been reported in the rabbit (13, 18) and in the adult rat (6, 19, 20, 21) and in cases of thyroid insufficiency in man (22–25). Even where increased size of eosinophiles is reported (17) there is evidence of degranulation and vacuolization. Vacuolization of the chromophobes has also been found, associated with an increase in the number and size of these cells, in thyroidectomized animals. The extra chromophobes were thought by Marine, et al. (18) to be degranulated eosinophiles. Severinghaus (26) has shown that they have the acidophilic type of Golgi apparatus, which, along with the characteristics of the mitochondria, indicates regression. That this return of the eosinophiles to the non-active chromophobe state is quite different from an active degranulation has been pointed out by Severinghaus (27). Whereas active degranulation might signify increased secretion, this type of change would seem to indicate a decrease in secretory activity.

Study of the basophiles has produced varied results and interpretations. Kojima (19) and Bryant (13) report a decrease in the number of 'ordinary' basophiles. Severinghaus, et al. (21) consider the vacuolated basophiles, which he later (27) calls thyroidectomy cells, as increased in number and similar to castration cells. However, according to Severinghaus (27), vacuolation occurs much more rapidly and extensively after thyroidectomy than after castration. Bryant (13) in rabbits, Schooley (17) in pigeons, and Grobstein (28) in Triturus torosus interpret the vacuolation in the pituitary following thyroidectomy as an atrophic change. Schooley (17) reports atrophy of the testes in such animals. Other workers (5, 29, 30, 31) also claim that the function

of the reproductive apparatus is reduced following thyroidectomy. These changes might result from a decreased potency of the pituitary or might be a direct effect on the gonads themselves. In the latter event the pituitary would be expected to show not a decreased but an increased potency similar to that in a castrated animal. Engle (32) and Guyer and Claus (33) considered the vacuolated basophiles of castrate animals as probably actively secreting. Severinghaus (27) has pointed out that the Golgi apparatus in these cells is shrunken, both in castrate and in thyroidectomized animals, that all castrates do not develope vacuolated basophilic castration cells, and all do show a marked increase in the size and number of granular basophiles. He has suggested that the increased gonad-stimulating potency of the pituitary in castrates is more probably dependent upon the presence of this latter type of cell. In thyroidectomized rats the granular basophiles, even though initially increased in number, soon show degenerative changes which become more extensive with time (20, 26). Guyer and Claus (33) also consider the basophiles as degenerate in thyroidectomized rats. Thus the cytological picture of the basophiles, as well as that of the eosinophiles, indicates a hypofunctioning gland following thyroidectomy.

Injecting thyroxin or feeding desiccated thyroid gland to adult rats (34) has also resulted in an increased size and vacuolation of the basophiles. The vacuolation is much less extensive, however, than that following thyroidectomy. The acidophiles on the contrary show marked hypertrophy of Golgi apparatus and mitochondria and an increased granulation, changes indicative of increased secretory activity.

Experiments designed to test the gonad-stimulating potency of pituitaries of hyperthyroid animals have in the main given positive results. Thus Evans and Simpson (1), Cohen (35), Van Horn (4) and Reforzo Membrives (6) found that in male and female white rats fed thyroid the gonad-stimulating potency of the pituitary, as tested by implantation into immature female rats, was increased. Van Horn reported this increase to range from 15 to 63%. Smelser (5), on the other hand, found no evidence of increased pituitary potency in the male rat and showed that hyperthyroid rats had lower testis weights and accessories similar to those of castrates (36).

Similar tests of pituitaries of thyroidectomized animals have not given consistent results. Evans and Simpson (1) and Van Dyke and Ch'En (2) have described decreased gonadotropic potency using female rats and rabbits, respectively, as the donor animals. More recently Reforzo Membrives (6) and P'An (7) have also reported decreased gonad-stimulating potency following thyroidectomy in normal male rats (6, 7) and in male and female rabbits (7). The former found no difference between castrate rats and those which had been both castrated and thyroidectomized. In P'An's rats there was a decrease in the latter group also. Smith and Engle (3), Van Horn (4), and Smelser (5), on the other hand, found little or no change in the response of ovaries of immature mice and rats to pituitary implants from thyroidectomized as compared with normal donor male and female rats. Smith (37) states that the administration of thyroid increases the gonad-stimulating potency of the hypophysis but that thyroid deficiency does not reduce it: Okano and Tanaka (8) report that thyroidectomy actually increases the gonad-stimulating potency of the pituitary.

In this study tests of the pituitaries, made by implantation into immature female mice, resulted in an increase in the average weights of ovaries and uterine tubes as compared with those of control animals. In the hosts to pituitaries from hypothyroid donors ($\mathcal{T}$) in *series 1* the increase was somewhat less in both ovaries and tubes than in those which received implants from normal animals (P hosts). The differences between the two sets of hosts are small; that of the ovaries slightly less than 2 times its probable error, that of the uterine tubes slightly more. However, taken in connection with the greater number of animals with a closed vagina in the $\mathcal{T}$ as compared with

the *P* series, they may perhaps be considered as indicative of a slight decrease in potency of gonad-stimulating hormone. The results from *series 2* are similar but more definite and consistent and the differences are significant. The decrease in gonad-stimulating potency of the pituitaries of hypothyroid animals is approximately 70% as compared with the potency of glands of normal donors. This increased effect over that found in *series 1* may be due to greater proficiency in technic, to the use of extracted powder of the pituitaries instead of fresh glands, or to the longer period of injection; 5 injections over a period of 5 days were used in *series 2* as compared with 1 or 2 injections over a period of 4 days in *series 1*. The use of a larger number of donor pituitaries per host should aid in the detection of the difference but it was done in too few cases to be able to judge. It is interesting in this connection that Evans and Simpson (1), who report a decrease in gonad-stimulating potency of the pituitary following thyroidectomy, used 2 glandular implants per host rat per day for 3 days which was a larger dosage than that of Van Horn (4) who obtained negative results with similar hosts. Other investigators reporting no change in potency fail to mention the exact dosages used. Reforzo Membrives (6) used fresh implants of 3 rat pituitaries per host, 2 the first day and 1 the second, and killed the animals on the fourth day. His hosts were 24-day-old rats; 10 received pituitaries from donors thyroidectomized 30 to 92 days previously, and 8 from normal donors; 5 littermate controls received no treatment. The average weights of host ovaries were 50.6, 74.3, and 27.6 mg. respectively. The decrease in gonad-stimulating potency due to thyroidectomy was 47% for his animals. If the data from *series 1* and *2* of this study are averaged they show a corresponding decrease of 36%, if *series 2* is considered alone the decrease is 65%. P'An's (7) results showing a 53% decrease are comparable. He also used rat hosts, giving a total dose of 4.8 mg. of pituitary ground up and injected subcutaneously in a 1:3,000 merthiolate solution, 8 equal portions over a period of 4 days. Here the dosage was smaller but the method of injection may have allowed for a more complete test.

The histological examination of the ovaries of *series 1* gave more consistent results than their weights. All of the litters studied showed a greater increase in the percentage of vesicular plus luteinized follicles in hosts receiving implants from normal donors than from hypothyroid donors. Therefore, if the percentage of vesicular and luteinized follicles is an indication of the potency of injected pituitary, it would seem that the pituitaries of hypothyroid donors are less potent in gonad-stimulating hormone than those of normal donors. The average total number of follicles was also greater in hosts to normal donors. According to Lane (9) FSH increases the total number of follicles, LH makes them vesicular, FSH matures them and LH exerts a luteinizing effect. The lower percentage of vesicular and luteinized follicles in hosts to hypothyroid donors might be considered indicative of a greater reduction to LH than of FSH. Reforzo Membrives (6) found no luteinization in the hosts to thyroidectomized donors, but an intensified effect from thyroid-fed animals. He also reports a less degree of follicular maturation in the hosts of the latter group than in hosts to glands from untreated donors. These facts would seem to indicate an increased production of LH after thyroid feeding. However, if the change in the basophiles following thyroidectomy is an atrophic one, then one would espect to find a decrease in FSH. Hellbaum and Greep (38) maintain that implants of pituitaries of normal males show very small amounts of LH, measurable only if large dosages are used. The decrease in gonad-stimulating potency in the male gland as tested in this study would then more likely be a decrease in FSH. The smaller size of the follicles noted by the authors and by Reforzo Membrives (6) would indicate such a decrease. The similar picture in the histology of the basophiles after thyroid feeding and thyroidectomy might, on the

other hand, result in the former from inhibition due to increased LH (39) if, as has been suggested (26), the basophiles are the source of FSH and the eosinophiles of LH. The explanation of the varying results of different investigators of thyroid-pituitary interrelationship may lie in the interactions, direct or indirect, between these two gonadotropic hormones.

SUMMARY AND CONCLUSIONS

Fifty-five young male white rats (21–28 days of age) were thyroidectomized and after 21 to 50 days their pituitaries were studied histologically, or were injected into immature female mice 18 to 20 days of age. Littermate female mice received glands of normal male donors that were littermates of the thyroidectomized animals, or were left untreated. The hosts were given 1 to 3 pituitary glands and were killed on the 4th and 5th day after the first injection.

Thyroidectomy almost invariably caused an increase in the weight of the pituitary. Histological examination showed a decrease in the number and size of eosinophiles with a corresponding relative increase in the number of chromophobes and basophiles. Vacuolization was especially marked in the chromophobes and basophiles.

The ovaries and uterine tubes of injected hosts showed an increase in weight over those of littermate controls. For hosts receiving pituitaries of thyroidectomized donors the average increase was less than for those injected with glands from normal donors.

Histological examination of 6 sets of host ovaries showed a smaller average total number of follicles and a consistently smaller percentage of vesicular plus luteinized follicles in animals injected with pituitaries of thyroidectomized donors than in those receiving glands from normal donors. The difference was approximately equal to that found in hosts to thyroidectomized donors compared with normal uninjected control mice.

The data indicate a decrease in gonad-stimulating potency of the pituitary of the young male rat 21 to 50 days after thyroidectomy.

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DOES A STIMULATION OF THE GERMINATIVE EPITHELIUM OF IMMATURE MALE RATS OCCUR AFTER TREATMENT WITH GONADOTROPIC HORMONES, AUGMENTATIVE SUBSTANCE, ANDROGENS AND VITAMIN E?[1]

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IN JANUARY 1926 ZONDEK and Aschheim (1) reported the stimulating effect of the anterior lobe of the hypophysis on female gonads. This was independently described by Smith and Engle in November 1926 (2). Gonadotropic hormone produces in the infantile ovary specific stimulation phenomena known as the anterior pituitary reactions I–III: follicle maturation, follicle hematoma and corpus luteum formation, respectively.

The effect of gonadotropic hormone stimulation in infantile male mammals, however, is not clear and the reports are contradictory.

Smith and Engle (3) have implanted anterior pituitary lobes on 3 consecutive days into immature male rats, and have obtained an enlargement of the accessory sexual glands but no premature spermatogenesis. In conformity with these results are those reported by Fels (4) who, on injection of pregnancy serum (prolan+estrone) obtained an enlargement of the interstitial tissues of the testicle, but a reduction in the germinative epithelium, apparently as a consequence of the antagonistic effect of the estrone injected.

B. Zondek, H. Zondek and Ucko (5), did not obtain precocious maturity following a single implantation of the anterior lobe of the hypophysis into infantile male rats. Similar results were reported by B. Zondek and Aschheim (6).

The advances in the purification of prolan (7) made it possible to obtain gonadotropic hormone in a concentrated form, and thus to renew the experiments on the stimulation of infantile male sex organs, especially in mice and rats. The use of mice and rats in similar experiments is a disadvantage perhaps since the male gonads of these animals seem to react very irregularly to gonadotropic hormone.

The experiments of B. Zondek and Aschheim (8), and those of Boeters (9) with prolan, have shown that there is premature descent of the testis and an enlargement of the accessory sex glands following a primary increase in the interstitial tissue of the testis. No accelerated spermatogenesis was observed. Only after employment of large doses of prolan did a precipitate maturation of the germinative cells take place; this finally led to the formation of giant-cells, abnormal nuclear mitosis and regressive changes. There was no premature regular spermatogenesis. These results are in agreement with those reported by most of the other authors (3, 10–31).

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The fact that some authors have reported complete spermatogenesis in infantile animals shows however that this problem is not a simple one. They (32–42) have reported successful stimulation of spermatogenesis with formation of spermatozoa, following injections of gonadotropic preparations in infantile male animals and in men.

Wells and collaborators (43–45) succeeded in obtaining early sexual maturity with gonadotropic hormone in ground squirrels. Moreover, hypophysectomized ground squirrels reacted with descent of testes and sperm production on administration of gonadotropic extracts (46).

Successful stimulation of spermatogenesis in hypophysectomized rats with pituitary gonadotropic hormone is also reported (20, 47–51). The majority of authors point out that no regeneration, but maintenance of fertility, follows the treatment with gonadotropic hormone when this is begun soon after hypophysectomy. Smith (52) was unable to confirm these reports with regard to hypophysectomized apes. He did not succeed in preventing the atrophy of the testes by the use of gonadotropic or adrogenic hormones.

As has been shown by Allanson, Rowlands and Parkes (53) prolan has no influence on spermatogenesis in the ferret during the anestrous season. However, the testes may be activated through 47 days of illumination and the last stages of development brought about by prolan (cf. Bissonnette (54).)

It seems possible that the failure of testis stimulation with gonadotropic hormone in most of the experiments is due to a disproportion in the prolan A and B content of the extracts. As has been demonstrated (55–58) *factor A* (FSH) stimulates the development of the germinative epithelium of the testes while *factor B* (LH) promotes the development of Leydig's interstitial tissue. The interstitial tissue produces androgenic hormone which in large doses inhibits the hypophysis of the experimental animals (18, 59–62). An indirect inhibition of the generative epithelium of the testes may thus be caused by a surplus of *factor B*. The effect of gonadotropic hormone on the male gonads may be manifested in the following ways. *a*) Direct effect of *factor A* on the germinative epithelium, *b*) interaction of the androgenic hormone secondarily produced by *factor B*.

Small amounts of androgenic hormone have a stimulating influence upon the hypophysis, whereby spermatogenesis is secondarily promoted (44, 45, 63). Large quantities of androgenic hormone inhibit the hypophysis and thus also spermatogenesis (18, 59–67, and unpublished observations).

It is possible that after removal of the hypophysis the mechanism of testicular stimulation is mediated through the suprarenal or thymus glands or even directly. Androgenic hormone is unable to regenerate the atrophic testes of hypophysectomized rats (27, 28). When, however, the androgen treatment is commenced immediately after hypophysectomy in mature rats, spermatogenesis may still be preserved (28, 52, 68–75). In hypophysectomized immature rats even testicular descent and spermatogenesis may be brought about if androgen treatment starts immediately after the operation (62, 69).

The fact that the ovaries of infantile animals may be brought to full function by chorionic gonadotropin, while it is impossible to provoke spermatogenesis in the testes shows that a fundamental difference exists between the reaction of these two gonads. This suggests that the maturation of the testes seems to require the co-operation of a substance which is unnecessary in the maturation of the ovaries. This special substance or gland seems to be present in young male birds, since they react to pituitary extracts with precocious maturity (76–80).

The manifold reports and contradictory results of the experiments described above induced us to study once more the mechanism of stimulation of the male germinal glands, since this is important from the clinical point of view.

EXPERIMENTAL METHOD

The experiments were performed on rats of an albino breed raised in this country for the last 15 years.

The stimulating effect of the following substances was studied. 1. Gonadotropins from pregnancy urine,[2] pregnant mare blood,[3] anterior pituitary,[4] menopause urine,[5] male urine.[5] 2. Testosterone.[6] 3. Vitamin E.[7] 4. Serum of mature cocks.

The effects of these substances were examined separately as well as in combination. We chose cocks as serum 'donors' because pituitary gonadotropin brings about precocious maturity in cockerels, which does not take place in other mammals. It is possible that an unknown susbstance supporting maturation of the testes circulates in bird-serum. Besides, the combination of cock serum and pituitary gonadotropin effects a certain augmentation of gonadotropic activity (Maxwell effect).

The experiments were performed on a total of 130 infantile male rats. The gonadotropic experiments were begun at 20 days of age (weight about 20 gm.) and were continued for 10 days (30 days of age and an average weight of 30 gm.). The rats were killed with ether narcosis and the sex organs removed in toto. After 24 hours' fixation in 10% formalin the testes and epididymes were weighed and examined histologically. The increase in weight of the testes alone is no indicator of a stimulation of the generative tissue because such an increase may be caused by an increase in the interstitial tissue.

Five to ten animals of different breeds were used for each experiment. Corresponding littermates were employed in the control experiments. In the control animals we generally found complete spermatogenesis on the 50th day of life. By means of hormonal stimulation it was attempted to produce this state of maturity as early as the 30th day. The experimental animals received a standard diet rich in vitamins and minerals. Vitamin E was also given daily to all animals in the form of 2 drops of concentrated wheat germ oil (60 × conc). We sometimes gave vitamin E in corresponding doses subcutaneously instead of orally whereby, however, the results were not affected.

The gonadotropic preparations were injected in solutions of 1.0 cc. of water or in cock serum. We have first standardized these preparations in infantile female rats, and the amount required to produce vaginal estrus after 5 days was considered 1 R.U.[8] (81, 82). The doses given below all refer to this unit. 1 R.U. was chosen as the smallest daily dose. As an average daily dose we used that amount required to produce luteinization (between 5 and 10 R.U.), and as the highest daily dose, 100 R.U. High doses inhibited spermatogenesis through hyperstimulation of Leydig's interstitial tissue.

Testosterone was injected in doses of 1 to 10 mg. in oily solution and sometimes rubbed on the scrotum in corresponding amounts of propylenglycol. However, the

[2] Korotrin of the Winthrop Chemical Company, New York City.
[3] Gonadogen of the Upjohn Company, Kalamazoo, Michigan.
[4] Ambinon of Roche-Organon Inc., Nutley, N. J.
[5] Gamone and Prospermine of Squibb and Sons, New Brunswick, N. J.
[6] Testosterone of Schering Corporation, Bloomfield, N. J.
[7] Viteolin of the Glaxo Laboratories.
[8] In chorionic gonadotropins this corresponded to ⅓ I.U.

percutaneous method of application gave the same results as those obtained with subcutaneous administration.

RESULTS

The experimental results are reported in table 1. The results are summarized in average values because they did not differ qualitatively even in optimal experimental

TABLE 1. REACTION OF 20-DAY-OLD INFANTILE MALE RATS TO 10 DAYS OF TREATMENT WITH GONADOTROPIC HORMONES, COCK SERUM, TESTOSTERONE AND VITAMIN E

Number of rats	Cock serum daily 10 days	Testosterone daily 10 days	Average weights of rats		Descensus, age	Average wt. of sex glands on 30th day of life		Histological reaction of the testes	
			20th day of life	30th day of life		testes	epidid.	Increase of interstit. tissue	Spermiogenesis in the tubuli
	cc.	mg.	gm.	gm.	days	mg.	mg.		
Controls receiving cock serum and testosterone									
30	—	—	20	30	—	156	42	—	2–3 layers
10	1	—	19	31	—	160	40	—	2–3 layers
10	—	1–10	19	29	29	390	117	±	5–7 layers
Pregnancy urine prolan, 5–100 R.U.									
10	—	—	19	30	26	250	75	±	2–3 lyaers
Pregnant mare blood gonadotropin 1–100 R.U.									
5	—	—	22	32	25	420	125	+	5–7 layers
10	1	—	20	30	24	450	110	+	5–7 layers
Anterior pituitary gonadotropin, 1–25 R.U.									
10	—	—	22	30	28	300	100	±	5–7 layers
5	1	—	19	28	27	450	110	±	5–7 layers
10	1	1–10	20	32	28	610	157	+	5–7 layers (sperm heads in 2 rats receiving 10 mg. of testost. daily
Menopause urine gonadotropin, 10 R.U.									
5	—	—	19	31	28	290	99	±	5–7 layers
10	1	1–10	22	32	26	501	136	±	5–7 layers
Male urine gonadotropin, 10 R.U.									
5	—	—	18	30	28	285	95	±	5–7 layers
10	1	1–10	22	31	27	498	132	±	5–7 layers (sperm heads in 5 rats which received from 6 to 10 mg. testosterone daily

conditions, i.e., in no case was complete spermatogenesis obtained. Certain quantitative differences were observed in the various preparations used. These are reported in table 1 and are as follows. The slightest degree of testicular stimulation was the formation of 5 to 7 layers of germinal epithelium in the tubules with numerous mitoses, while the controls normally had only 2 to 3 layers of primitive resting cells. The highest degree of stimulation observed in our experiments was the formation of sperm heads. This may be noted as the beginning of spermatogenesis, but it is still far from being a complete spermatogenesis with mature spermatozoa. This latter stage was never attained in our stimulation experiments.

We were unable to ascertain any relation between descensus testiculorum and spermatogenesis, especially because the descensus began earlier when high doses of gonadotropic hormones were employed (100 R.U.). Acceleration of spermatogenesis

and increase in weight of the testes was more marked with average doses (10–20 R.U.) especially if anterior pituitary gonadotropin and male urine gonadotropin were used.

DISCUSSION

These experiments demonstrate that gonadotropic preparations of various kinds and in combination with an androgenic factor or an augmenting factor such as cock serum were unable to bring about spermatogenesis in infantile white rats receiving vitamin E. It is not clear why cocks become precociously mature through pituitary gonadotropin, while rats are hardly affected. The injection of cock serum into rats did not overcome this refractory state. Nevertheless, we have obtained in a number of cases the formation of sperm heads, but never any complete spermatogenesis. The 2 series in which sperm heads were formed on the 30th day of life were in white rats which received daily *a*) anterior pituitary gonadotropin (10 R.U.)+cock serum (1 cc.)+testosterone (10 mg.). Two rats treated in this way both reacted with sperm head formation. *b*) male urine gonadotropin (10 R.U.)+cock serum (1 cc.)+testosterone (6–10 mg.). Five rats treated in this way (6, 7, 8, 9 and 10 mg. of testosterone) reacted with sperm head formation.

Since sperm head formation was never found in the control rats before the 40th day we may say that by the above two combinations we succeeded in bringing about a certain, though limited, acceleration of spermatogenesis. These results support the supposition that the process of maturation of the young testes is a complex process, perhaps a chain type of reaction, in which several other factors take part besides the gonadotropic hormone. The mammalian testis needs, therefore, a longer period of maturation. Further experiments should be made in order to ascertain a means of testes stimulation by various combinations which will successfully bring about premature spermatogenesis.

The refractoriness of infantile mammalian testes to gonadotropic hormone is in sharp contrast to the response of the ovaries of mammals which readily react to gonadotropin with the three anterior-pituitary reactions.

SUMMARY

The influence of pregnancy urine prolan, pregnant mare blood gonadotropin, anterior pituitary gonadotropin, menopause urine gonadotropin and male gonadotropin on spermatogenesis has been studied. In albino male rats, 20 days old, richly fed with vitamin E, complete spermatogenesis was never produced with these hormones in a 10 day period of treatment.

The augmentation of these gonadotropic preparations with cock serum also gave no acceleration of spermatogenesis. A certain acceleration of spermatogenesis but no sperm formation was obtained with testosterone if anterior pituitary gonadotropin with cock serum or male urine gonadotropin with cock serum were administered. These two combinations lead to the formation of sperm heads in a part of the experimental rats at the 30th day of life.

The administration of other gonadotropic preparations as pregnancy urine prolan, pregnant mare blood gonadotropin, menopuase urine gonadotropin in combination with cock serum and testosterone did not bring about any acceleration of spermatogenesis.

These experiments support the supposition that spermatogenesis in mammals is a complex process in which, besides the hormones so far studied, several still unknown factors are involved. Precocious maturation of the ovaries is a relatively simple process involving the use of gonadotropin only.

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FAILURE OF STEROID HORMONES TO INDUCE MAMMARY GROWTH IN HYPOPHYSECTOMIZED RATS

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SINCE THE FIRST REPORT THAT mammary glands of hypophysectomized rats failed to grow when stimulated by estrogen injections (1) others have appeared denying and confirming this observation (for review of literature, Riddle, (2)). The majority of evidence is in favor of the view that pituitary secretions or certain pituitary extracts, notably those containing growth hormone (3, 4, 5), are essential for mammary stimulation by estrogens. Several hypotheses have been mentioned to account for the pituitary-sex hormone-mammary gland-growth relationships. One suggests that estrogen stimulates the pituitary to induce growth by the secretion of a pituitary "mammogen" (6, 7, 8), which is thus an indirect action of the steroid hormones. Another group believes that the "nutritional state" of the animal will either prevent or permit the mammary-stimulating action of estrogens (9). Evidence has been presented to support the contention that estrogens can act directly on the mammary gland when the hormone is applied in the form of an ointment directly to the skin overlying the gland (10, 11, 12). To these we have added our concepts (5) that the pituitary does possess and secrete a mammogenic factor which is capable of acting synergistically with estrogen to induce mammary growth. Furthermore, this does not invalidate the concept that the pituitary by itself can induce new mammary growth (13).

In this report, we wish to show that substitution of steroid hormones for the hypophyseal mammogenic factor is without effect on mammary growth and that the hypophyseal factor seems necessary for estrogen stimulation.

METHODS

Young male rats weighing 85 to 140 gm. were hypophysectomized and after a lapse of time or immediately after the operation, they were injected with various combinations of steroid hormones. Our choice of combinations was limited to desoxycorticosterone acetate, testosterone proprionate, and estradiol diproprionate.[2] The effects of these hormones so admirably reported in normal animals by numerous investigators gave us an adequate basis for comparsion. Hypophysectomized females were also used in experiments to test the effect of direct application of estrogens to the skin over the mammary glands. Treatment periods were usually from 12 to 20 days, administrations being made once daily.

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[2] Desoxycorticosterone acetate (Percorten), testosterone proprionate (Perandren); estradiol diproprionate (Di-Ovocylin). Estradiol for inunction (Ovocylin) supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

The mammary glands were stained and mounted in toto, the right abdominal gland being used in each case. Great care was exercised in examining the sella turcica for completeness of extirpation. The anterior part of the brain was lifted upwards and with a sharp pointed scissors first the optic nerves and then the pituitary stalk were cut near the base of the brain. In this way, a piece of the stalk was always left to be sectioned along with the capsule and, as we discovered, there was less chance for a small piece of hypophyseal tissue to escape detection.

EXPERIMENTAL RESULTS

Our first study was to determine if desoxycorticosterone would, by itself or in combination with estrogen, induce new mammary growth in the absence of the hypophysis. Twenty-two rats were divided into 2 experimental groups, one of which

TABLE 1. INFLUENCE OF STEROID HORMONES ON MAMMARY GROWTH IN HYPOPHYSECTOMIZED RATS

Exp. no.	Experimental treatment	Days post-operative	Av. change in body wt.	No. rats	Pituitary	
					Absent, no growth	Present, with growth
			gm.			
1	Desoxycorticosterone 6 mg. in 12 days	5	+7	4	2	2
	Desoxycorticosterone+120γ estrogen	5	−13	5	2	3
2	Desoxycorticosterone 7.5 mg. in 15 days	35	+6	6	5	1
	Desoxycorticosterone+150γ estrogen	35	−10	7	7	0
3	Testosterone 8 mg. in 16 days	56	−8	8	8	0
4	Testosterone 5.5 mg. in 12 days	0	−7	7	7	0
	Testosterone+200γ estrogen	0	−12	8	7	1
5	Testosterone 12.5 mg. in 20 days	0	0	7	5	2
	Testosterone+10 mg. desoxycorticosterone	0	+15	7	6	1

received treatment 5 days after the operation, and the other, 35 days later. Those rats which received the desoxycorticosterone acetate alone gained in body weight while those which received estrogen in addition lost weight (disregarding those which were shown to have some pituitary tissue remaining). In none of these 16 cases receiving desoxycorticosterone acetate alone or in combination with estrogen was there any evidence of mammary growth (table 1). The glands presented the usual picture of a reduced naked duct system characteristic of the hypophysectomized rat.

In contrast to this, whenever a remnant of anterior hypophyseal tissue was found, new growth in some of the end buds was observed. The effect of the desoxycorticosterone acetate was like that of estrogen alone in partial hypophysectomy (5). Of particular interest was one rat in *experiment 2* which had a small piece of poorly vascularized pituitary remaining and showed no evidence of mammary stimulation. The other endocrine weights (thyroid, adrenals, and testes) indicated a complete absence of the hypophysis.

The reports of mammary growth resulting from testosterone treatment in normal rats (14) and the fact that such treatment seems to be beneficial to hypophysectomized rats in general led us to study this hormone alone and in combination with estrogen and desoxycorticosterone acetate. Rats which were hypophysectomized for 56 days and then received treatment with testosterone did not show mammary growth in a single instance (table 1, *experiment 3*). The body weights of the animals decreased

as much as 22 gm. or gained as much as 8 gm. yet no effect on the mammary glands was noted.

The combination of estrogen with testosterone (immediate treatment) likewise failed to show evidence of any new growth of the end buds which is so characteristic of estrogen treatment in normal male rats. While no difference among the mammary glands could be observed as a result of the treatments, yet when compared to those in uninjected controls, it appeared that the glands had not regressed as rapidly as if no treatment had been given. This was revealed particularly in the central parts of the gland where many groups of alveoli could still be seen in spite of marked involution of the rest of the structures.

A combination of testosterone and desoxycorticosterone acetate likewise failed to affect the mammary gland any differently from either one alone, (table 1, *experiment* 5). The mammary glands, as above, had not regressed as extensively as in the uninjected controls. The rats which received the combined injections gained weight remarkably well, averaging 15 gm. in 20 days.

The experiments of others designed to show the direct effects of estrogen on the mammary gland when applied to the skin have not ruled out the possible rôle played by the circulating mammogenic factors since normal animals have been used. Therefore, this work was repeated on hypophysectomized rats. Four female rats were castrated and hypophysectomized for 21 days. The hair on the right side of the lower abdominal region was shaved and doses of approximately .04 mg. of estradiol[2] in the form of a paste were thoroughly rubbed into the shaved area on alternate days for 21 days. Frequent shavings were necessary to remove the renewed hair growth. The mammary glands of the treated side in each rat were compared with those of the untreated side.

The results showed that in no case could the gland on the right treated side be distinguished from the other. Two of the females had no growth of the mammary glands at all and were completely hypophysectomized. The other 2 had well developed end buds and new growth but they also had small remnants of hypophyseal tissue. The hormone administered in this way was sufficient to affect the uterus and vagina, probably because the rats could lick the hormone from the skin.

This experiment was repeated on 4 hypophysectomized and 3 normal young male rats. Instead of using the hormone paste, a small amount of an oil solution of estradiol di-proprionate was dissolved in an alcohol-ether mixture and doses of .05 cc. (0.1 mg.) applied to the shaved area daily for 19 days. Care was taken that the drops covered only one side and by blowing on the skin the solvent was quickly evaporated.

The results again showed that no mammary growth occurred in the 4 completely hypophysectomized males but excellent growth occurred in the 3 normal animals. In the normal animals the treated and untreated glands could not be distinguished from each other.

DISCUSSION

Substitution of certain steroid hormones for the hypophyseal mammogen failed to induce mammary growth in the hypophysectomized rat. Desoxycorticosterone, by itself or in combination with estrogen was incapable of stimulating new growth. Previously (15), it had been shown that desoxycorticosterone acetate could stimulate mammary growth in normal and castrated male mice and our own observation with partial hypophysectomy confirms this for the rat. Gomez and Turner (7) tried a similar experiment in hypophysectomized guinea pigs and likewise failed to stimulate the mammary glands.

Testosterone, which is capable of inducing mammary growth in the normal or

castrated male rat (14), failed to do so in the absence of the hypophysis. This also confirms the work of others, particularly Noble (16) and McEwen, Selye and Collip (17). When treatment with testosterone or combinations with the other steroid hormones was begun immediately after the operation, the glands did not regress quite so rapidly as when no treatment was given. In this respect, the maintenance action of testosterone on the mammary glands is similar to that of maintenance of the testis.

Combinations of testosterone with desoxycorticosterone acetate were of special significance to us in view of the concept of the relation of body growth to mammary growth. Astwood, et al. (9) believe that the poor nutritional state of the rat which results from hypophysectomy accounts for the failure of estrogen to stimulate mammary growth. Desoxycorticosterone acetate in the operated rat apparently improved the nutritional state as revealed by an increase in body weight. Since this substance in the normal animal induces new mammary growth one might expect a similar result in the hypophysectomized rat. This was not the case, however, as definite slow regression of the mammary tree set in.

Further evidence for the necessity of some pituitary factor for mammary growth was revealed in the experiments on direct application of the hormone to the skin over the glands. In no case, in the absence of the hypophysis, was new growth seen. In the normal or partially hypophysectomized animals the localized influence of the applied hormone was not observed and the glands of the treated and untreated sides showed equal stimulation. This localized effect might be a species difference, since mice, rabbits and monkeys were used by other workers (10, 11, 12). It is more likely, however, that the rats licked the excess hormone from the abdominal surface and this systemically stimulated both glands equally.

Though our results seem to indicate that the pituitary is indispensable for renewed growth of the mammary glands of the rat, Gardner recently has reported that certain of the steroid hormones alone or in combination will induce mammary gland growth in the hypophysectomized male mouse (18). Estrogen alone in small doses was capable of inducing mammary growth, although previously it was reported by Gomez, Turner, Gardner and Hill (19) that no growth resulted when large doses were employed. Combinations of desoxycorticosterone acetate or progesterone with estrogen gave the best results in the hypophysectomized mice. Apparently the mouse and rat react differently under similar circumstances.

Further evidence that steroid substances are ineffective is demonstrated by the recent work of Greep and Stavely (20), who showed that lipoid solvents could not remove any fraction from pituitary tissue which would induce growth of the mammary gland of hypophysectomized rats. This is opposed to the findings of Lewis and Turner's (21) work in which they report that pituitary mammogen is lipoid-soluble.

In the concept of the pituitary-sex hormone-mammary gland-growth relationship, we feel that a number of factors may contribute to the final product of a fully formed mammary gland. Of these, we wish to stress the importance of the pituitary mammogen (which has not as yet been isolated) acting alone and synergistically with the sex hormones to bring about normal growth responses.

SUMMARY

Desoxycorticosterone, testosterone and estrogen, either alone or in combination will not induce new growth in the mammary gland of the hypophysectomized rat. Testosterone seems to slow the rate of mammary gland involution which follows hypophysectomy. The administration of estrogen directly on the skin over the mammary gland of the hypophysectomized rat is without effect, although in the normal or partially hypophysectomized rat, new growth is stimulated. These experiments

further the concept that pituitary mammogen is essential for steroid hormone effects on the mammary glands of hypophysectomized rats.

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MAMMARY GLAND DEVELOPMENT WITH MAMMOGEN I IN THE CASTRATE AND THE HYPOPHYSECTOMIZED RAT[1]

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IN 1936, REECE et al. (1) published the first experiments showing that the mammary glands of completely hypophysectomized animals did not respond to administration of ovarian hormones. Gomez and Turner (2) soon demonstrated that the anterior pituitary gland from pregnant animals or those pretreated with estrogen contained a factor which would cause mammary development in hypophysectomized or normal guinea pigs. It was also demonstrated that thyrotropic, adrenotropic, and lactogenic AP extracts or adrenal cortical extract plus estrogen were ineffective in hypophysectomized guinea pigs. The theory was then advanced by Gomez and Turner (3) that a previously unrecognized pituitary factor or factors were the direct agents of mammary growth. This complex was called mammogen. From earlier observations with ovarian hormones it appeared that there must be two mammogenic factors. The duct growth factor (*mammogen I*) was thought to be stimulated to increased secretion by estrogen alone and caused mammary duct growth. The second factor (*mammogen II*) was assumed to be stimulated to secretion by estrogen plus progestin during pregnancy or pseudo-pregnancy, causing growth of the mammary lobule-alveolar system.

The experimental evidence concerning the growth of the mammary gland following ovarian hormone administration in the hypophysectomized animal was reviewed by two of the present authors in 1939 (4). At that time positive results had been reported in the rat (5–8), the rabbit (9), the guinea pig (10), and the dog (11). These reports all appeared before 1936. On the other hand, since 1935, negative results have been reported in the rat (1, 2, 12, 13, 14), the guinea pig (2, 15, 16), in the mouse (2, 17, 18), and in the rabbit, cat, and ground squirrel (2). Several additional publications on this question have since appeared.

Lacassagne and Chamorro (19) stated that they could obtain no mammary development in hypophysectomized mice given intraperitoneal injections or subcutaneous pellets of estrogen.

Selye (20) stated that maintenance of the mammary gland is under the control of pituitary mammogenic hormones. A large variety of hormones cause mammary growth because they all have the property of stimulating the anterior pituitary gland to produce increased amounts of mammogenic hormones. Hypophysectomized rats showed no mammary development after treatment with large doses of progesterone which were adequate to cause con-

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siderable development in normal rats. Hypophysectomy did not adversely affect the action of large doses of progesterone on other secondary sex organs.

Treatment of hypophysectomized rats with estrogen plus progesterone was ineffective or the response was greatly reduced, according to Uyldert et al. (21). In castrate rats this treatment caused extensive development. They concluded that estrogenic, progesterone-like and pituitary factors are necessary for complete mammary development.

Herold and Effeckmann (22) introduced another aspect into the problem by claiming that estrogen is ineffective on the mammary glands, as well as on the other sexual organs, in rats with the hypophyseal stalk cut. This was refuted by Dempsey and Uotila (23). The latter reported that in such animals reproductive cycles, ovulation, mating, pregnancy, and lactation were all normal unless the anterior pituitary gland was injured during the operation.

Estradiol benzoate at 1000 I.U. daily did not produce growth of the mammary glands of hypophysectomized rats, according to Nathanson et al. (24). Even when pituitary growth extract was added to the treatment, mammary glands of hypophysectomized rats which gained weight were similar to those of untreated controls. In other rats of the group the glands were atrophied. The authors concluded that although nutrition was of some importance the sex hormones must act through the pituitary.

Samuels et al. (25) approached the question of the effect of nutrition on the growth of the mammary glands which had been first raised by Astwood et al. (14); this was done by forced feeding of rats with a stomach tube. Five gm. of thyroid was fed daily to raise the metabolism. Salt and cortical extract supplemented the decreased adrenal activity. Force-fed hypophysectomized rats given 1000 I.U. of estradiol benzoate every other day for 28 days showed no mammary development. Controls similarly treated had considerable mammary growth. The authors concluded that either the change in metabolism caused by anterior pituitary gland extract is necessary for growth or that estrogens act through the anterior pituitary gland. They suggested that Astwood's results on undernourished rats were due to the sensitivity of the pituitary to inanition. Production of mammogen may then be curtailed.

Reece and Leonard (26) also found that estrogens were unable to cause mammary growth in hypophysectomized rats. Endogenous estrogen, presumably produced following the administration of pregnancy or menopause urine extracts or FSH, was also ineffective. A growth hormone extract caused slight development in hypophysectomized males. With estrogen added to the growth extracts appreciable development occurred. There appeared to be a correlation between the increase in weight of such animals and mammary growth stimulation. The thyrotropin or adrenotropin in the growth extract was ineffective. It was suggested that either estrogen facilitates the action of the mammogenic factor in the growth extracts or vice versa.

In opposition to these reports which support the idea of an indirect action of ovarian hormones on the mammary glands, Frederickson (27) reported that estradiol benzoate or this hormone plus progestin would cause growth of the mammary glands of hypophysectomized immature rabbits. He states, however, that these changes were not apparent on gross examination; usually only on microscopic examination at high magnification were growth changes apparent. He writes of intense arborization of ducts and lobule-alveolar development in glands 1 to 2, and 2 to 3 cm. in diameter. All sellae turcicae were serially sectioned. Frederickson also obtained complete mammary development in does made pseudo-pregnant and then hypophysectomized. The corpora lutea were maintained by estrogen injections and caused mammary lobule-alveolar development.

Gardner (28) also reported slight mammary growth in a small proportion of hypophysectomized male mice given desoxycorticosterone acetate, progesterone, or estradiol dipropionate. When estradiol was combined with desoxycorticosterone or progesterone, a higher proportion of mice responded and to a greater extent.

The question of the direct action of estrogens has also been approached by application of hormone to the skin over the mammary glands. Jadassohn et al. (29) and Zondek (30) reported unilateral mammary development in guinea pigs by percutaneous application, as did Lyons and Sako (31) in the rabbit, Gardner and Chamberlain (32) in mice, and Speert (33) in the monkey. MacBryde (34) claimed unilateral increase in size of treated breasts in women. Chamberlain et al. (35) applied estrone to one breast of male monkeys. Although externally

the treated breasts appeared much enlarged and the nipples were developed, the mammary glands showed little development over those of the controls. The latter authors considered the local action of estrogens, which two of us have confirmed in the rat and rabbit (unpublished data), to be critical evidence of the direct action of estrogen on the mammary gland. The present writers believe there is some doubt of this. The estrogens are known to produce hyperemia and to increase metabolism when locally applied (36, 37). Lewis and Turner (4) have shown that a certain level of mammogen is present in the anterior pituitary of cattle, in all stages studied. An increased blood flow and metabolism in localized areas might readily aid the normal blood mammogen in producing localized mammary hyperplasia.

The possibility of producing growth of the mammary glands with anterior pituitary tissue and extracts has been extensively studied. Schultze and Turner (38), Weichert et al. (39), and Loeb and Kirtz (40) were unable to produce growth of mammary glands with implantation of individual pituitaries in the mouse. Gomez and Turner (2) emphasized the use of anterior pituitary tissue from pregnant or estrin-treated animals. They were successful in the use of anterior pituitary tissue from pregnant cattle in the rat, rabbit, and guinea pig as was Nelson (41, 42) with anterior pituitary tissue plus estrogen in the rat. Gomez and Turner (2) reported that pretreatment of donor animals with estrogen caused the production of a mammogenic substance in the anterior pituitary since mammary glands developed in hypophysectomized and castrate guinea pigs which were given daily implants from such donors; pituitaries from untreated rats were ineffective. Nelson (42, 43), and Reece and Leonard (44) also obtained mammary growth in hypophysectomized rats with pituitary tissue but could detect no difference between pituitaries from estrogen-treated and normal donors. Reece and Leonard concluded that this was evidence for a pituitary mammogenic factor while Nelson disagreed. It is quite possible that the mammary gland of the hypophysectomized rat is not suitable for demonstrating the quantitative difference in mammogenic content of pituitaries from estrogen-treated and normal donors. Lewis and Turner (4) with the aid of their male mouse assay technique for mammary duct growth were able to show considerable differences between the *mammogen I* content of anterior pituitaries from estrogen treated and normal rabbits. Male rabbits given 300 to 700 I.U. of estrone daily for 10 days had 65 to 100% more *mammogen I* in their pituitaries than 10-day-pregnant rabbits. Gomez and Turner (3) obtained similar results in female rabbits when 3 times the anterior pituitary dosage from normal untreated male rabbits and 4 times as much anterior pituitary from females gave no mammary growth in the male mouse. Anol, and anol plus estradiol also increased the *mammogen I* content of female rabbit anterior pituitary, apparently more than did estradiol alone (45).

Lewis and Turner (4) reported an extensive investigation of the mammogenic duct growth potency of cattle anterior pituitary using the male mouse assay technique. The *mammogen I* content of anterior pituitary glands from pregnant dairy and beef cows was found to be low in early and late pregnancy, rising to a peak at the 50th day. This curve of *mammogen I* content did not at all resemble the output of estrogen during pregnancy as shown by Turner et al. (46).

The *mammogen I* content of dairy cow anterior pituitary gland was higher than that of beef cows throughout pregnancy. Dairy cows, of course, produce considerably greater quantities of milk than beef cows and have larger, more fully developed mammary glands. Non-pregnant cows were found to have an appreciable amount of *mammogen I* in the anterior pituitary gland though less than in pregnancy. The content was highest during lactation. Dry cows with corpora lutea in the ovaries had less than lactating cows but twice as much as dry cows with only ovarian follicles.

By far the highest content of *mammogen I* was found in the anterior pituitary glands of adolescent heifers with corpora lutea. During this stage of heifer development the mammary duct system is actively proliferating. Anterior pituitary tissue from steers, bulls, and fetuses contained measureable amounts of *mammogen I*.

These studies involved the treatment of 1033 mice with anterior pituitary substance at dosages in the neighborhood of one M.U.; 350 responded with mammary duct growth. Some 320 pituitaries from cattle of known stages of development were assayed in groups of from 1 to 42. Two large lots of pituitaries from pregnant cattle obtained from a packing plant were

also assayed. This anterior pituitary material was found to have little or no estrogenic activity at the mammogenic M.U. dosage. Stimulation of duct growth in spayed female and a male rabbit was also reported with fresh anterior pituitary tissue from pregnant cattle.

Gomez and Turner (2) reported development of mammary lobule-alveolar systems in rats and rabbits by injection of AP from pregnant cattle. Lewis and Turner (4) secured similar results in spayed mice with AP as did Nelson (41) in rats given AP plus estrogen. Mixner et al. (47, 48) secured lobule development in a large series of spayed mice given anterior pituitary from pregnant cattle. This anterior pituitary tissue was found to contain no progesterone when assayed by the sensitive McGinty technique (49).

Growth of mammary glands with AP extracts involves the problems of the content of the original tissue in mammary growth hormones and the question as to whether or not the extraction procedure has been suitable for the preservation and concentration of the factors. Only recently have assay techniques been available for quantitative estimation of the amount of mammogenic duct growth (4) and lobule-alveolar growth (47, 48) factors. These techniques have so far been used only in the Missouri laboratory.

A number of investigators have reported negative results on mammary gland development with AP extracts. These may be explained on the basis of the inactivity of the particular extracts or insufficient dosage. Corner (50) in 1930, on the other hand, reported that fresh alkaline extracts of anterior pituitary caused full mammary development and lactation in young mature ovariectomized rabbits. The mammary growth and lactation factors were unstable and elusive. These results were confirmed by Asdell et al. (51) except that immature rabbits were found to be unresponsive. Lyons and Catchpole (52, 53) claimed that the lactogenic hormone would produce growth of the duct and lobule-alveolar system of rabbits. Lyons has since found that purified lactogenic AP extracts will not cause growth of the mammary lobule-alveolar system in rats (54). A crude AP extract plus estrogen caused extensive duct and lobule development in hypophysectomized rats (13) as did AP extract in spayed or hysterectomized mice (55).

Astwood (56) reported that whole pituitary powder caused or allowed proliferation of the lobule-alveolar system of pseudopregnant hypophysectomized rats. With the use of lactogenic-adrenotropin fractions of pituitary there was little or no proliferation. There was no correlation between proliferation of the mammary glands and maintenance of the corpora lutea. Some luteotropic extracts caused lobule formation, others did not. Crude placental extracts were also effective.

Extraction procedures for *mammogen I* have been reported by Lewis and Turner (4). They found that acetone drying caused a 62% loss of potency in anterior pituitary tissue from pregnant cattle. Extraction with ethanol at PH 9 to 10 and precipitation at PH 5.7 plus ether-drying of the precipitate caused a loss of 95% of the mammogenic potency. Unlike most of the other anterior pituitary hormones, *mammogen I* was found to be soluble in lipid solvents and was not precipitated by adjustment of the PH. It was necessary to avoid high temperatures during extraction and to employ freezing temperatures for storage, for the lability reported by Corner (50) and Asdell (51) was confirmed. A number of potent extracts have been obtained requiring 0.1 to 1.0 mg. per male M.U. Investigation of the estrogenic potency of these extracts gave negative results at 1.24 to 10 times a male M.U. by the vaginal smear technique. One extract showed a slight amount of estrogen by the Astwood (57) technique. Lipid extracts of the anterior pituitary which were high in duct-growth potency caused no lobule-alveolar development in spayed female mice (4, 47, 48). Since fresh anterior pituitaries from pregnant cattle were positive for lobule development, a separation of two mammary growth factors had apparently been obtained.

Greep and Stavely (58) confirmed the presence of a mammary growth factor in fresh anterior pituitary tissue using hypophysectomized castrate rats. By their method of extraction, which entailed considerable heating, they apparently lost all mammary growth factor from the lipid fraction but were able to obtain growth with the protein fraction. Using the rat they were unable to estimate yields on a quantitative basis or to differentiate between duct growth and lobule-alveolar factors.

Some investigators believe that the adrenals may have a direct influence on mammary growth. Gomez and Turner (2) did not obtain mammary growth in normal, incompletely, or

completely hypophysectomized guinea pigs with desoxycorticosterone. Desoxycorticosterone acetate was reported by Van Heuverswyn et al. (59) to cause duct growth in male mice; two of us have confirmed this (unpublished data). Speert (60) claimed lobule-alveolar growth in mature spayed monkeys with the same treatment. Dantchakoff (61) reported mammary development in rats treated from birth with desoxycorticosterone acetate plus testosterone. Chamorro (62) reported that mammary glands of hypophysectomized mice showed no signs of development after treatment with desoxycorticosterone acetate although control mice readily responded. Gardner (28) did not confirm this, obtaining a small proportion of responses in hypophysectomized mice and better results when desoxycorticosterone acetate was given with estradiol.

Gomez and Turner (2) obtained no mammary development in hypophysectomized guinea pigs with adrenotropic extract of pituitary alone or in combination with estrogen. Nelson (63) found that an adrenotopic extract of pituitary caused slight to marked mammary growth in immature male and female rats. This also occurred in gonadectomized and in hypophysectomized rats with some variation in the female. If both the gonads and adrenals were removed the results were negative. In the female the effects were so striking in some cases that Nelson suggests that both estrogen and progestin action must be produced from the adrenals. Butcher (64) reported that in underfed, adrenalectomized rats the mammary glands grew faster than in littermates with adrenals. Spaying did not affect these results.

The objects of the following experiments were to extend to the castrate male rat the study of mammary duct growth with lipid AP extracts, and to show that active mammary-duct proliferation can be instituted in the hypophysectomized rat with such extracts.

PROCEDURE

Male albino rats of various ages were castrated and allowed to recover for a week or more in order to eliminate from the mammary glands all signs of growth due to normal hormonal relations in the body. They were then injected subcutaneously with one of three lipid extracts of AP from pregnant or non-pregnant cattle. The daily dosage was administered in 0.1 to 0.2 cc. of oil in which the extract had been dissolved. The castrate rats were fed ad libitum a mixed, ground grain ration which contained cod liver oil.

Twenty-five to 44-day-old albino rats were hypophysectomized by Dr. Gomez and were then treated with a *mammogen I* extract supplied by Dr. Lewis and Dr. Turner. This extract was shipped special delivery, packed in CO_2 ice. It was adequately refrigerated upon arrival at Beltsville, Md. The rats were hypophysectomized an average of a week before treatment was begun. Control mammary glands were removed from several rats. The *mammogen I* was dissolved in oil. The daily dose was administered in a single subcutaneous injection. The sellae turcicae were serially sectioned after autopsy and examined for residual fragments of pituitary.

The mammary glands were prepared as whole mounts by a method described by Turner and Gomez (65), slightly modified.

Extract 45 was prepared by warm ether-alcohol extraction of anterior pituitary tissue from pregnant cattle and ether fractionation as described by Lewis and Turner (4). Approximately 0.23 mg. was equivalent to 1 M.U. of *mammogen I*. *Extract 61* was prepared by acetone-ether extraction of anterior pituitary tissue from non-pregnant cattle as described by Bergman and Turner (66) in the acetone-drying of AP, plus ether fractionation. It contained 1 M.U. of *mammogen I* per mg. *Extract 60* was obtained in the same manner from anterior pituitary glands of pregnant cattle; 0.1 mg. was equivalent to 1 M.U.

The thyroids, adrenals, gonads and uteri of the hypophysectomized rats were weighed at autopsy. The significance of the difference between the average weight of

these glands in the treated and control female groups was calculated by the formula $Sd = V(1/n_1 + 1/n_1)$ as given by Snedecor (67).

The first experiment had as its object the determination of the amount of *mammogenI*, assayed in the mouse, required to stimulate the growth of the mammary gland of the castrate rat. As the male rat mammary gland is well developed in comparison to that of the mouse, it is necessary to stimulate considerable growth in order for it to be readily observable in comparison to the control.

RESULTS

Negative results on mammary duct growth were obtained in 17 castrate male rats given *mammogen I* at the rate of 0.02 to 1.6 M.U. in 6 injections (table 1). On the

TABLE 1. GROWTH OF THE MAMMARY GLAND IN THE CASTRATE MALE RAT WITH AP MAMMOGENIC DUCK GROWTH EXTRACTS

Body wt. autopsy, gm.	No. of rats	Extract no.	Dosage/6 days, M.U.	No. of injections	Results
78	3		untreated	0	Slight or no regression 2 wk. after castration
148	3	Olive oil	1.8 cc.	6	Regressed
98	3	45	0.02	6	No duct development
142	2	45	0.1	6	No duct development
102	3	45	0.2	6	No duct development
100	5	45	0.4	6	Appeared active. No duct end-buds
91	2	45	0.8	6	Appeared active. No duct end-buds
80	2	45	1.6	6	Appeared active. No duct end-buds
92	4	45	0.04	28	1 positive; end-buds
96	4	45	0.1	30	1 positive; end-buds
102	4	45	0.2	30	1 positive; end-buds
111	2	45	0.8	30	1 positive; end-buds
106	2	45	1.6	30	1 positive; end-buds
122	2	45	3.2	30	Both positive. Much duct development
206	3	61	12.0	16, 28, 39	1 positive; end-buds
260	3	61	24.0	16, 28, 39	1 positive; end-buds
338	3	61	48.0	16, 28, 39	1 positive; end-buds
95	3	60	300.0	20	3 positive

larger dosages the mammary glands appeared to be in an active condition, showing no regression of lobule development but no duct end-buds were present. There may have been some lobule development in these glands for this is difficult to judge unless it is extreme since this is only a quantitative change. Control castrate male rats also showed some lobule development at this age.

Apparently a 6-day period was insufficient time for the development of duct end-buds in the castrate male rat since at least 1 of each of 5 groups of castrate rats showed end-buds at autopsy after treatment for a longer period (fig. 1). Groups of 2 to 4 rats were injected at the rate of 0.04 to 3.2 M.U. of *mammogen I* per 6 days, for 28 to 30 days. At the highest dosage both rats had duct end-buds and showed considerable duct development.

Three groups of 3 castrate male rats were given 12, 24 and 48 M.U. per 6 days of another lipid extract of *mammogen I*. A rat was killed from each group after 16, 28 and 39 injections. Only 1 rat from each group had duct end-buds at autopsy. Mammary glands from the other rats appeared active and may have had some lobule devel-

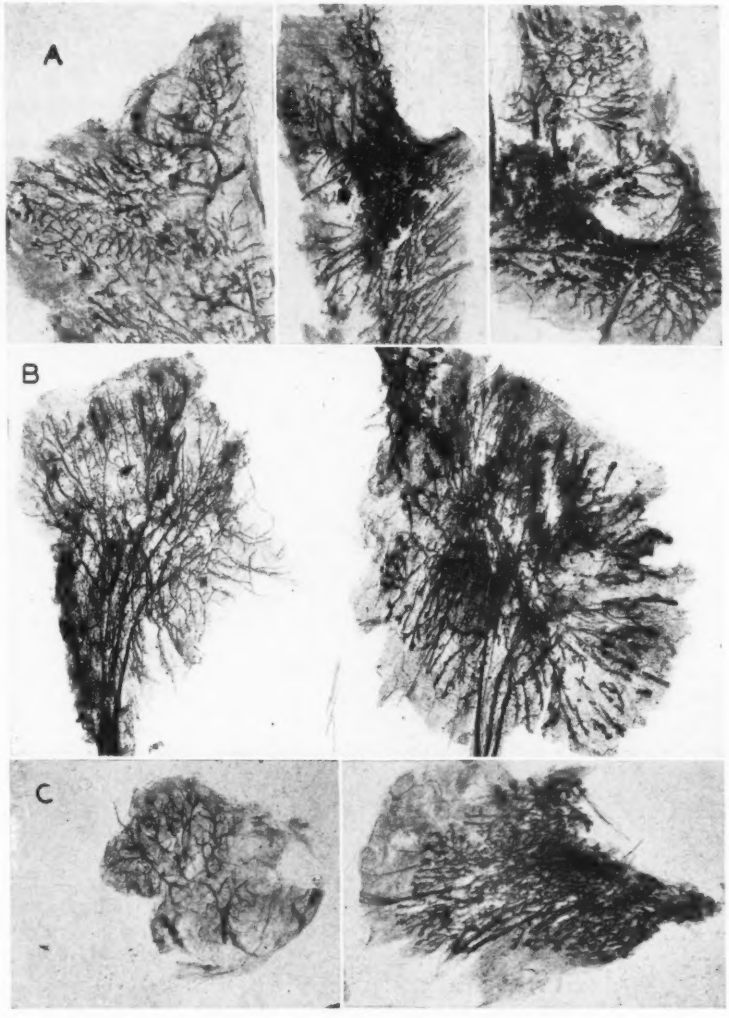

Fig. 1. A. MAMMARY GLANDS FROM 3 CASTRATE MALE RATS GIVEN LIPID EXTRACT OF CATTLE ANTERIOR PITUITARY. Two glands show considerable lobule development in the center of the glands which is typical of sexually mature male rats. The peripheral ducts are plain in each case. Duct end-buds are apparent showing active proliferation. ×4. *B. Left*, control mammary gland from a hypophysectomized female rat. *Right*, mammary gland removed from the same rat after 10 days' injection of a total of 40 M.U. of *mammogen I*, AP extract. Notice the strong proliferation of duct end-buds. ×4. *C. Left*, control gland quite atrophied. *Right*, mammary gland from the same hypophysectomized female rat after injection of a total of 80 M.U. of *mammogen I* extract. ×4.

opment. The number of injections had little effect on the response in these groups for one rat was positive for end-buds in each autopsy group.

Another group of 3 castrate male rats was given a very active *mammogen I* extract of anterior pituitary tissue from pregnant cattle. After 20 daily injections at the

TABLE 2. EFFECT OF MAMMOGEN I EXTRACT ON THE MAMMARY GLANDS, THYROIDS, ADRENALS, GONADS AND UTERI OF HYPOPHYSECTOMIZED RATS

Age at operation, days	Days rest	Wt. at injection, gm.	Dose/day, M.U.	No. of inj.	Autopsy					
					Body wt., gm.	Thyroid, mg.	Adrenal, mg.	Gonad, mg.	Uterus, mg.	Mammary gland response
Males, treated										
40	6	104	4	7	84					Positive
30	10	68	4	10	75	7.5	7.0	328		Positive
25	8	38	8	10	38	6.0	8.0	74		Positive
27	5	80	16	10	78	8.0	11.0	78		Positive
Males, untreated										
30	7	108	0		108	8.0	16.0	260		Not recov.
27[1]	7		0			12.0	18.0	96		Not recov.
40	7	125	0.1 cc. olive oil	10	125	12.0	16.0	360		Ducts atrophic
Females, treated										
30	10	62	4	10	64	5.0	5.5	8.0	28	Positive
30	10	82	4	10	88	6.5	7.0	4.5	26	Positive
25	8	50	4	10	66	6.0	9.0	10.0	37	Negative
30	7	72	8	10	72	7.0	8.0	9.0	32	Positive
30	7	82	8	10	86	7.0	7.5	8.0	36	Positive
30	7	72	8	10	88	5.0	8.0	9.0	41	Positive
30	7	80	8	10	86	8.0	10.0	20.0[2]	58[2]	Positive
27	5	48	16	10	48	8.0	10.0	13.0	29	Positive
27	5	74	16	10	72	8.0	10.0	9.0	23	Positive
27	5	72	16	10	72	9.5	10.0	11.0	26	Positive
40[3]	5	80	8	10	75	8.0	12.0		46	Positive
40[3]	5	80	8	10	78	10.0	12.0		42	Positive
Av. 31	7	70	9	10	74.6	8.4	9.1	10.2	35.4	
Females, untreated										
40[3]	5	88	0		72	10.0	10.0		36	Ducts atrophic
44[3]	5	—	0		78					Ducts atrophic
40	6	104	0		100	10.0	16.0	14.0	38	Ducts atrophic
25	8	74	0		74	10.0	18.0	20.0[2]	51[2]	Ducts atrophic
27	8	52	0		62	8.0	12.0	8.0	22	Ducts atrophic
30	7	92	0.1 cc. olive oil	7	75					Ducts atrophic
27	5	52	0		52	9.0	11.0	9.0		Ducts atrophic
27	5	60	0		60	7.5	9.0	6.0	24	Ducts atrophic
Av. 32	6	75			72	9.1	12.7	11.4	28.5	
t						0.81	2.75	0.52	0.97	
25[1]	8	62	4	10	79	15.0	19.0	30.0	79	
25[1]	8	43	8	10	63	18.0	25.0	24.0	68	
6 normal control 27–30-day-old rats[4]						24.5	32.6			
18 normal control 30-day-old rats[4]								17.0	67.4	

[1] Hypophysectomy was incomplete.
[2] The ovaries appeared to contain corpora lutea.
[3] Ovariectomized.
[4] Not hypophysectomized.

rate of 300 M.U. per 6 days all showed extensive mammary duct development and end-buds (table 1).

From this study it is seen that 3 lipid extracts of anterior pituitary, which required 0.1 to 1.0 mg. per *mammogen I* M.U., were effective in causing duct growth in castrate male rats when given for 16 to 30 days. Only negative results were secured with 6 daily injections. Positive results were secured at dosages as low as 0.04 M.U. per 6 days but the percentage response was not high until several M.U. a day were given.

The most important part of this study was that conducted by Dr. Gomez on hypophysectomized rats with an AP lipid mammogenic extract supplied by the Missouri laboratory. Twenty-nine young male and female rats were hypophysectomized and either treated with *mammogen I* after a rest period of 5 to 10 days or used as controls (table 2). Four male rats were given 4 to 16 M.U. of *mammogen I* extract per day for 7 to 10 days. All showed well-developed mammary duct systems with end-buds at autopsy.

Twelve hypophysectomized females were given similar dosages of *mammogen I* extract after a rest period of 10 days. Eleven of the 12 showed well developed mammary duct systems with end-buds at autopsy after 10 injections (fig. 1). Seven similar hypophysectomized untreated female rats had atrophic duct systems as did 1 given 0.1 cc. daily of olive oil.

These rats were well cared for after operation. The treated females gained 13.6 gm. per rat while the control group lost an average of 3 gm. The thyroids of the treated group were slightly, though not significantly, smaller than those of the control group. The adrenals of the treated group of females were significantly smaller than those of the control group. The value of t for the data was 2.72 which is slightly larger than statistical significance which requires a t of 2.12 at the 5% level with 16 degrees of freedom (67). This would indicate the possibility that treatment with *mammogen I* extract caused a decrease in weight of the adrenals of hypophysectomized female rats over that of similar controls. The difference in average weight of the ovaries between the treated and the control group was not significant. The uteri of the treated hypophysectomized females averaged 25% heavier than those of the controls. One rat in each group had ovaries twice as large as the average. These ovaries apparently contained corpora lutea. The uteri of these two rats were also enlarged. The variation in weight of the uteri of other rats in the group was also large so that the difference between average weights of the two groups was not significant. The average weight of the thyroids, adrenals, ovaries and uteri of both hypophysectomized groups was considerably under that of similar organs from normal control rats of the same age.

DISCUSSION

Previous work in the laboratory of the University of Missouri has indicated the growth-stimulating property of lipid extract of the anterior pituitary upon the mammary duct system of the male mouse. The present observations indicate that the mammary gland of the castrate male rat is similarly influenced, but, as would be expected, the dosage requirement for response is higher.

The more important question, however, is the influence of this extract upon the growth of the mammary gland of the hypophysectomized animal. Certain investigators have suggested that the reason for the ineffectiveness of estrogen in hypophysectomized animals is the low food consumption of such animals. The observations of Samuels et al. (25) oppose this idea since by forced feeding they were able to maintain the body weight of their hypophysectomized animals, yet showed that estrogen was

without effect. In this laboratory, Trentin and Turner (68) have further shown that estrogen will not stimulate duct growth in underfed mice as readily as in full fed animals, yet with increasing amounts of estrogen the effects of progressive inanition can be overcome.

The present data contribute to this problem in yet another way. It was shown that a pituitary extract, relatively free of the proteinaceous AP hormones as indicated by the lack of response of the ovaries, thyroid and adrenals of the hypophysectomized animals as well as by direct assay of the AP metabolic hormones (4), directly stimulated the growth of the mammary duct system of these animals. Thus without replacement therapy which might be expected to increase the nutritive condition of the experimental animals, mammary growth took place.

If *mammogen I* acts through one of the endocrine organs such as the adrenals, as has been suggested, it would be expected to cause an increase in size of the intermediary organ. The evidence presented here is against the thyroids, adrenals, ovaries and uteri having any part in mammary duct growth following the administration of lipid *mammogen I* extracts.

SUMMARY

A lipid *mammogen I* extract of pregnant-cattle anterior-pituitary glands gave only negative results in 6 injections into castrate male rats at total dosages of 0.02 to 1.6 mammogenic M.U. Positive results were obtained when treatment was continued for 28 to 30 days at dosages of 0.04 to 3.2 M.U. per 6 days. A lipid extract of cattle anterior pituitary caused duct growth at dosages of 12 to 48 M.U. per 6 days. A second extract of pregnant-cattle anterior pituitary gave strongly positive results at 300 M.U. per 6 days.

Sixteen 25 to 44-day-old male and female rats were hypophysectomized and treated with a lipid extract of pregnant-cattle anterior pituitary for 7 to 10 days. Fifteen showed evidence of mammary duct growth at autopsy. Dosages ranged from 4 to 16 M.U. per day. Ten hypophysectomized rats served as controls. The weights of the thyroids, adrenals, ovaries and uteri of the treated hypophysectomized female rats were compared with those of similar untreated rats. Only in the case of the adrenals was the difference significant. Adrenals from treated rats were smaller than those from controls. Average weights of all of these organs in both groups were much less than similar weights from normal unoperated control rats.

These data show that lipid AP extracts will cause growth of the mammary duct systems of castrate male and hypophysectomized male and female rats.

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CYCLICAL VAGINAL RESPONSE TO THE DAILY ADMINISTRATION OF ESTRADIOL IN CASTRATED RATS

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NEARLY ALL OF THE PHYSIOLOGICAL phenomena which occur in the female genital tract have in common a cyclical rhythmicity. This is clearly seen in the comparative study of the physiology of reproduction concerning which T. Martins (1) has said: "in sexual life all is rhythm." The regulation of this rhythm was attributed at first to the ovary, but later it was shown that it was possible to produce cyclical phenomena in the receptor organs of castrated animals receiving daily and constant injections of estrogens in adequate dosage. This demonstrates the importance of the receptor organs, the uterus and vagina, which present periodic fluctuations in their respective thresholds of response from causes which are still not well understood.

Del Castillo and Calatroni (2) demonstrated that daily injections of estrogens during a period of several months to female castrated rats produced the periodic appearance of vaginal estrus separated by diestrus lasting several days; the length of the diestrous periods were in general determined by the dose employed. From these experiments the conclusion was reached that some extra-ovarian factor regulates the periodicity of estrus by decreasing the vaginal sensitivity to estrogens during the refractory periods. Zuckerman (3) repeated the work of Del Castillo and Calatroni in rats and observed the same phenomenon in castrated immature monkeys which were injected daily for a year with 100 I.U. of estrone; metrorrhagia was observed every 35 to 50 days. Emmens (4) confirmed the experiment in mice. These observations indicate that the sensitivity of the receptor to the estrogenic stimulus has periodic variations independent of ovarian influence, a physiological phenomenon of considerable interest which we have further analyzed in this work. Zuckerman (5) showed that hypophysectomy had no effect on the duration of the refractory periods in castrated rats receiving daily doses of estrogens. In 3 of 7 castrated rats bilaterally adrenalectomized and injected daily with estrogens, he observed cyclic changes but estrus was greatly prolonged; in the 4 remaining animals estrus was persistent without refractory periods. From his experiments, Zuckerman concluded that there was a definite relation of the adrenals to the sensitivity of the vagina in castrated rats receiving estrogens.

The present investigation consists of the study of the threshold of vaginal response in castrated rats receiving daily injections of estradiol. The different factors capable of modifying the response were varied such as the dose of estradiol injected, the extirpation of various endocrine glands (hypophysis and adrenals), and the injections of other hormones (progesterone and desoxicorticosterone).

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RESULTS

Vaginal response to estradiol. Twenty-eight white female rats weighing approximately 160 to 170 gm. were studied for 20 days; vaginal smears were made daily. At the end of this period they were castrated by the lumbar route and daily vaginal smears continued in order to confirm the period of permanent diestrus. Twenty-four days later the rats were divided into 4 groups of 5 animals each and a control group of 8 rats. Each group of 5 received daily subcutaneous injections of estradiol benzoate in sesame oil in a dosage of 0.5, 1, 5 and 20 international benzoic units (I.B.U.) respectively while 5 of the controls were injected with pure sesame oil and 3 remained untreated. The various groups received the following solutions

Solution A—1 cc. = 4 I.B.U. (*groups* 1 and 2).
Solution B—1 cc. = 40 I.B.U. (*group* 3).
Solution C—1 cc. = 80 I.B.U. (*group* 4).

The 1st group received 0.5 I.B.U. daily for 90 days during which time there was a single estrus in each of 2 rats, 4 estrus periods in one, and none in the remaining rat. Estrus lasted 1 to 2 days. The 2nd group received 1 I.B.U. daily for the same period; after a latent period of 2 days proestrus appeared followed by estrus which lasted 3 to 9 days and occurred approximately every 10 days. The 3rd group were injected with 5 I.B.U. daily for 90 days; after a latent period of 1 to 2 days, proestrus appeared followed by estrus lasting 8 days, but the activity of the vaginal epithelium continued after brief periods of metestrus and occasional short periods of diestrus. In this group refractory periods were followed by metestrus. The 4th group received 20 I.B.U. daily and presented a picture similar to that of the 3rd; the latent period was one day, followed by prolonged estrus which was interrupted by short metestrus. The controls remained in permanent diestrus.

The optimun dose of estradiol benzoate for the demonstration of refractory periods of the vagina was 1 I.B.U. daily. With larger amounts (5 to 20 I.B.U.) the diestrous phases were usually replaced by metestrus, while with smaller doses (0.5 I.B.U.) few if any periods of estrus occurred. Del Castillo and Calatroni (2) and also Zuckerman (5) noted the importance of correct dosage in studying the vaginal response.

Vaginal response and adrenalectomy. The effect of bilateral adrenalectomy on the vaginal cycle of rats has been widely discussed by different investigators; in our strain of rats we have been able to confirm the conclusion of Del Castillo (6) that the vaginal cycles are not interrupted by bilateral adrenalectomy.

The experiments in this section are divided into two parts; in the first the animals were observed for 4 months, in the second for only 45 to 50 days inasmuch as such factors as proper dosage and time of vaginal response had been analyzed in the first group. *Part* 1. Eight castrated rats were divided into 2 groups. *a*) the 1st group of 4 rats was injected daily with 0.5 I.B.U. of estradiol benzoate for 90 days. In 1 rat estrus occurred 4 times, in 2, once each, and in the 4th not at all. At the end of 3 months the 4 rats were adrenalectomized, the injections of 0.5 I.B.U. of estradiol being continued. After the operation vaginal smears revealed the appearance of more frequent and prolonged periods of estrus separated by periods of diestrus. *b*) The 2nd group consisted of 4 rats which received daily injections of 1 I.B.U. of estradiol benzoate for 90 days; 9 characteristic periods of estrus lasting 3 to 8 days and separated by periods of refractoriness appeared. At the end of 3 months 2 animals were adrenalectomized. Prolonged estrus lasting 18 to 21 days occurred. In the two controls the vaginal epithelium continued to respond cyclically.

Part 2. Twenty castrated rats were divided into 3 groups. *a*) The 1st group consisted of 8 animals which were injected daily with 0.5 I.B.U. of estradiol benzoate for

15 days at which time both adrenals were extirpated, the injections of estradiol being continued until the rats were autopsied 20 to 24 days after operation. None of the animals had an estrous period during the 15 days before adrenalectomy; afterwards, estrus appeared in 6 of the rats and lasted 3 to 8 days. *b*) The 2nd group consisting of 6 rats received daily injections of 1 I.B.U. of estradiol benzoate, and after 15 days the adrenals were removed; before adrenalectomy 2 periods of estrus separated by diestrus occurred in each of 2 rats, a single estrus occurred in each of 3 and persistent diestrus was present in 1 rat. After adrenalectomy prolonged estrus lasting 8 to 25

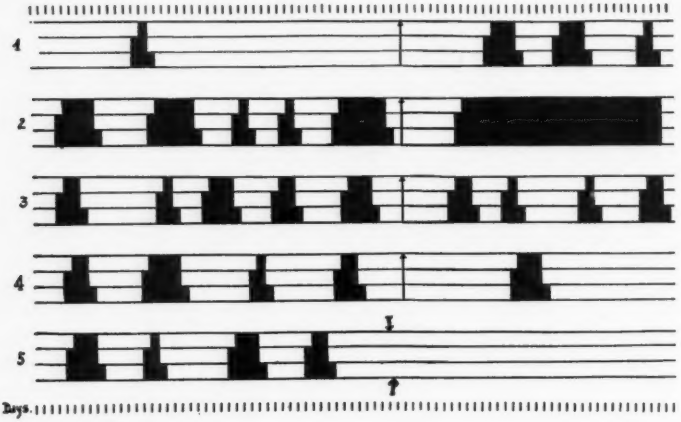

Fig. 1. RHYTHMIC VAGINAL RESPONSE OF CASTRATED RATS RECEIVING 0.5 AND 1 I.B.U. OF ESTRADIOL BENZOATE. 1. 0.5 I.B.U. daily; adrenalectomy, (*arrow*) estradiol injections continued. 2. 1 I.B.U. daily; adrenalectomy, (*arrow*) estradiol injections continued. 3. 1 I.B.U. daily; adrenalectomy (*arrow*), estradiol continued and in addition 0.5 mg. of desoxycorticosterone acetate daily. 4. 1 I.B.U. daily; adrenalectomy (*arrow*), estradiol injections continued and in addition 2 mg. of desoxycorticosterone acetate injected daily. 5. 1 I.B.U. daily; 2 mg. of desoxycorticosterone acetate daily (*arrow*). These rats were not adrenalectomized.

days appeared in all of the animals. *c*) The 3rd group consisted of 6 control non-adrenalectomized rats of which 3 received 0.5 I.B.U. and 3 1 I.B.U. of estradiol benzoate daily; the first 3 developed no estrous period for at least 42 days, while in those injected with 1 I.B.U. characteristic periods of estrus separated by refractory periods appeared.

The majority of adrenalectomized rats belonging to this Institute survive without the administration of cortical extract or sodium salts, although they show signs of insufficiency which reach their peak 6 to 12 days after operation; subsequently there is gradual improvement due to hypertrophy of accessory adrenal tissue the occurrence of which is constant (7) and becomes visible 3 to 8 weeks after operation.

With adrenalectomy and the minimal dose of estradiol benzoate (0.5 I.B.U.), there is an immediate appearance of estrus if this was previously absent, or more prolonged and frequent estrus periods in those cases in which estrus existed previously; always however, the cyclical response of the vagina was maintained. With the daily dosage of 1 I.B.U. of estradiol, adrenalectomy caused the appearance of prolonged periods of estrus. It is concluded that after adrenalectomy there is a greater sensitivity of the vagina of the castrated rat to repeated daily stimulation with estradiol.

Vaginal response and desoxycorticosterone. Because of the results obtained above, it was decided to investigate the action of desoxycorticosterone acetate to determine if it would prevent the lowering of the threshold of vaginal response provoked by

adrenalectomy. Robson (8) studied the action of desoxycorticosterone acetate on the vaginal cycle of the mouse and found that the administration of 0.1 to 0.2 mg. daily inhibited estrus in normal animals.

Twenty-five castrated rats weighing between 140 and 150 gm. were injected daily for 36 days with 1 I.B.U. of estradiol benzoate and the occurrence of estrus and periodic refractory periods were noted. At the end of this period the animals were divided into 4 groups. *a*) The 1st group consisted of 8 rats which were adrenalectomized, the injections of estradiol being continued; 3 rats died before the 8th day

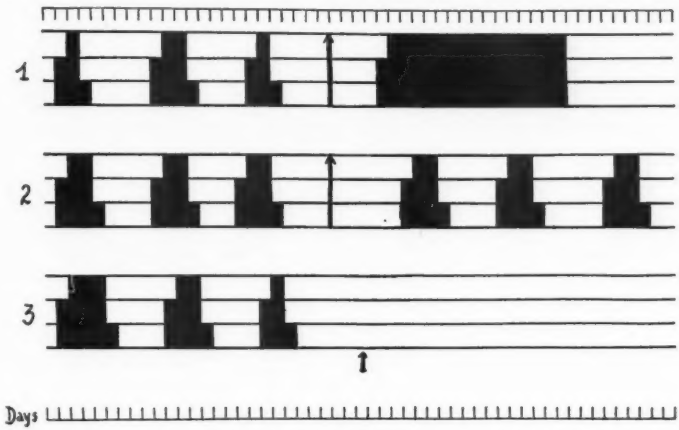

Fig. 2. RHYTHMIC VAGINAL RESPONSE OF CASTRATED RATS RECEIVING 1 I.B.U. OF ESTRADIOL BENZOATE DAILY. 1. *Arrow* marks adrenalectomy following which estradiol injections were continued. 2. *Arrow* marks adrenalectomy following which estradiol was continued and in addition 0.5 mg. of progesterone injected daily. 3. 0.5 mg. of progesterone (*arrow*) in addition to estradiol benzoate. These rats were not adrenalectomized.

and are therefore not included in the study. In the remaining animals continuous estrus appeared lasting 10, 16, 16, 17 and 17 days respectively. *b*) The 2nd group also consisted in 8 rats, adrenalectomized, to which the administration of 1 I.B.U. of estradiol benzoate daily was continued; in addition 0.5 mg. of desoxycorticosterone was injected daily. In these animals short periods of estrus continued to appear separated by refractory periods of diestrus similar to those which existed before the extirpation of the adrenals. *c*) The 3rd group consisted of 6 rats which were adrenalectomized and injected daily with 1 I.B.U. of estradiol benzoate and 2 mg. of desoxicorticosterone acetate; in these animals, short periods of estrus continued to appear and were less frequent than before the operation. The animals consistently gained weight which they did not do with 0.5 mg. of desoxycorticosterone. *d*) The 4th group was composed of 3 rats which were injected with 1 I.B.U. of estradiol benzoate like the others and 2 mg. of desoxycorticosterone acetate daily; these animals were not adrenalectomized. After the injection of desoxycorticosterone the vagina remained in the diestrous phase permanently in spite of the continued administration of estradiol.

From these experiments, it is concluded that the daily injection of 0.5 mg. of desoxycorticosterone prevented the continuous estrus provoked by adrenalectomy since short periods of estrus separated by refractory periods persisted. If the dose were increased to 2 mg. daily, estrus occurred more infrequently; and finally if 1 I.B.U. of estradiol benzoate and 2 mg. of desoxycorticosterone were injected daily into castrated rats with intact adrenals, the vagina remained in a permanent diestrous

phase. Desoxycorticosterone therefore, increased the vaginal threshold to estradiol and furthermore neutralized the effect of adrenalectomy on the vaginal sensitivity.

Vaginal response and progesterone. The influence of progesterone on the vaginal sensitivity to estradiol was studied in castrated rats with and without adrenals. Twelve rats weighing 150 gm. were castrated by the usual technic; 1 week later daily injections of 1 I.B.U. of estradiol benzoate were begun, and during the ensuing 18 days characteristic periods of estrus separated by periods of diestrus appeared. At the end of 18 days the animals were divided into 3 groups. *a*) The 1st group consisted of 4 rats which were adrenalectomized, the injections of estradiol being continued; in all of the rats prolonged estrus of 10 to 17 days occurred. *b*) The 2nd group consisted of 4 animals which were adrenalectomized and injected daily with 1 I.B.U. of estradiol benzoate and 0.5 mg. of progesterone; short periods of estrus separated by refractory periods continued. *c*) The 3rd group composed of 4 rats with intact adrenals were injected daily for 18 days with 1 I.B.U. of estradiol benzoate and 0.5 mg. of progesterone; in none of the animals did estrus appear, the vagina remaining permanently in the diestrous phase.

The daily injection of 0.5 mg. of progesterone prevented the continuous estrus provoked by adrenalectomy in castrated rats receiving daily injections of 1 I.B.U. of estradiol benzoate; if the animals had intact adrenals, progesterone abolished the vaginal response, leaving the vagina permanently in the diestrous phase.

Vaginal response and hypophysectomy. Since hypophysectomy produces atrophy of the other endocrine glands of the body, experiments were repeated in hypophysectomized rats in order to eliminate all hormonal action on the vagina, with the exception of the injected estradiol. Zuckerman (5) studied the vaginal response to estradiol in hypophysectomized castrated rats and demonstrated that the cyclical vaginal response continued. Twelve castrated rats weighing between 160 and 200 gm. were injected daily with 1 I.B.U. of estradiol benzoate for 23 days during which time the characteristic vaginal response was noted; at the end of this period hypophysectomy was performed in 8 of the rats by Dr. Foglia, the injections of estradiol being continued. The hypophysectomized rats lost weight but continued to show the same vaginal response as preoperatively, just as the controls; estrus was perhaps somewhat prolonged but it was always separated by periods of diestrus. The rats were killed 20 days after hypophysectomy and autopsy confirmed the absence of the hypophysis and an appreciable diminution in the weight of the adrenals.

DISCUSSION

The quality of rhythmic response is an innate characteristic of the rat vagina. This rhythmicity is correlated with ovarian function, but a rhythmic fluctuation of the ovarian secretion is not the sole factor as is shown in these experiments. If the castrate rat receives an adequate amount of estradiol benzoate daily the vaginal rhythm becomes evident, short estrous periods separated by diestrus occur notwithstanding the continuation of uninterrupted injections. The physiologic action of the ovary is similar to that of a constant amount of estrogen injected each day. In order to demonstrate the vaginal rhythm it is necessary to inject an adequate amount of estrogen daily; for estradiol benzoate this is 1 international benzoic unit (I.B.U.). If larger doses than this are used, there is a lengthening of estrus together with a shortening of the diestrus periods, and finally a state of prolonged estrus interrupted by fleeting periods of metestrus occurs.

We have investigated the question of the effect of rhythmical function of other organs on the vaginal rhythm. Adrenalectomy, which in our rats has no influence on the vaginal cycle, causes in castrated rats a greater sensitivity of the vagina to

estradiol, that is, it lowers the threshold of vaginal response. In the optimum dosage of 1 I.U. estradiol benzoate produces prolonged estrus after adrenalectomy and the refractory periods disappear. If a minimal dose of 0.5 I.B.U. is employed periods of estrus appear for the first time after operation if they were not present before, or they are more prolonged if they occurred previously. In summary it may be said that adrenalectomy reduces the threshold of vaginal response to estradiol. On the other hand the daily injection of desoxycorticosterone acetate or progesterone after adrenalectomy neutralizes the effect of this operation on the sensitivity of the vagina to estradiol, since the rhythm of estrus separated by diestrus persists. The fact that the adrenals influence the threshold of vaginal response to estradiol appears to be clear but we are unable to explain the intrinsic mechanism of its action. It must be emphasized again that with minimal doses of estradiol after adrenalectomy the cyclical response of the vagina becomes more manifest.

Progesterone and desoxycorticosterone acetate increase the threshold of vaginal response to estradiol since they abolish the estrus which is obtained with an adequate amount (1 I.B.U.) in the castrated rat. This cannot be interpreted as a direct neutralizing effect of the progesterone and desoxycorticosterone acetate on the estradiol, for if the injections are repeated in castrated adrenalectomized rats they do not suppress the vaginal cyclical response.

Hypophysectomy does not alter the vaginal sensitivity to estradiol within 20 days after the operation, for if estradiol injections are continued the vaginal cyclical response continues unchanged.

As a result of these experimental observations it appears that there is a law of cyclical vaginal response to estradiol, which may be expressed as follows. The vagina of castrated white rats possesses an intrinsic cyclical activity which is revealed by the repeated injection of estradiol, is abolished by the injection of progesterone or desoxycorticosterone, and is not influenced by hypophysectomy.

CONCLUSIONS

This report confirms previous work showing that castrated female rats receiving repeated, daily and adequate doses of estradiol benzoate have a cyclical vaginal response.

Bilateral adrenalectomy results in a greater sensitivity of the vagina of the castrated rat to continuous stimulation by estradiol benzoate.

Desoxycorticosterone acetate and progesterone decrease the vaginal sensitivity of castrated rats to continuous stimulation by estradiol benzoate. Desoxycorticosterone acetate and progesterone neutralize the effect of adrenalectomy on the sensitivity of the vagina to estradiol in castrated rats.

Hypophysectomy, within 20 days postoperatively, does not alter the vaginal sensitivity to estradiol in castrated rats.

The estradiol benzoate, progesterone, and desoxycorticosterone acetate used in this study were supplied by The Schering Corporation, Bloomfield, N. J. and Ciba Pharmaceutical Products, Inc., Summit, N. J.

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EFFECTS ON THE PERINEAL SWELLING AND ON THE MENSTRUAL CYCLE OF SINGLE INJECTIONS OF COMBINATIONS OF ESTRADIOL BENZOATE AND PROGESTERONE GIVEN TO BABOONS IN THE FIRST PART OF THE CYCLE[1]

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IT HAS BEEN SHOWN PREVIOUSLY (1) that 3 to 15 mg. of progesterone administered to baboons as a single injection in the first part of the cycle rapidly induced perineal deturgescence and rest within 4 to 7 days; without the intervention of bleeding the perineum commenced to swell once more and passed through a normal cycle except that the experimental cycle of which this new one was a part was lengthened. A single injection of as much as 20 mg. of progesterone, however, given under similar circumstances, induced the swollen perineum to return rapidly to rest within 4 to 7 days, but bleeding was precipitated prematurely and the cycle was shortened by more than half its normal length.

Moreover, when a single injection of 1 to 5 mg. of estradiol benzoate was given to baboons in the first part of the cycle the turgescent perineum continued to increase in size for 4 to 11 days and then slowly underwent deturgescence and perineal rest; without the intervention of bleeding a new but almost normal perineal cycle followed. The experimental cycle of which the new one was a part was lengthened by 15 to 20 days (2).

The object of this investigation was to discover whether the administration of combinations of estradiol benzoate and progesterone given concurrently would produce effects on the perineum and the menstrual cycle which could be attributed to estradiol or progesterone alone in accordance with the criteria established for each in previous studies or whether the reactions elicited could be regarded as a resultant of their combined effects. If the latter should prove to be the case then obviously it is necessary to determine how the original estrogen response was modified by progesterone and vice versa.

MATERIAL AND METHODS

All of the baboons used were adults whose cycles had been closely followed for at least a year by perineal measurements as previously outlined (3). In 4 instances the same baboon was used twice, but only several months after the first experiment and only when 3 consecutive cycles were normal.

The intramuscular injections of estradiol benzoate[2] and progesterone[2] were simultaneously administered on the 8th to the 10th day of the cycle.

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[1] This investigation was assisted by a grant made by the National Research Council and Board (South Africa).

[2] Estradiol benzoate (Progynon-B in oil) and progesterone (Proluton) were supplied by the Schering Corporation, Bloomfield, N. J., through the courtesy of Dr. Stragnell.

EXPERIMENTAL

First experiment (5 mg. of estradiol benzoate plus 20 mg. of progesterone; *baboons* 1 to 4, table 1). Three different perineal responses were elicited. *Baboons* 1 and 2 responded broadly in a similar fashion. Forty-eight hours after the injection the turgescent perineum underwent deturgescence (fig. 1). This was fairly rapid at first but later became more gradual. By the 9th day perineal rest had been reached and was maintained for 4 to 5 days. Thereafter without the intervention of bleeding the perineum commenced to swell gradually in *baboon* 1 and very rapidly in *baboon* 2. On the 32nd day of the cycle in the case of *baboon* 1, the perineum had attained its maximum dimensions and on this day bleeding unexpectedly occurred. Bleeding associated with a turgescent perineum is a feature which has *never* been observed in

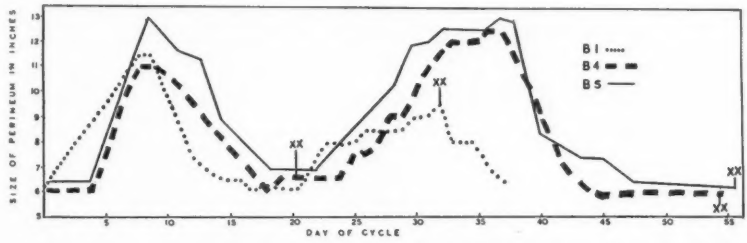

Fig. 1. Effect of combinations of estradiol benzoate and progesterone injected on the 8th day of the cycle upon the size of perineum and onset of bleeding in normal baboons. *B* 1 and *B* 4 received 5 mg. of estradiol benzoate and 20 mg. of progesterone, and *B* 5, 5 mg. of estradiol benzoate and 10 mg. of progesterone. XX denotes onset of bleeding.

normal baboons. In *baboon* 2 the maximum deturgescence was reached on *day* 26 which was earlier than in *baboon* 1. On the 32nd day when the perineum was 3 inches above the resting level bleeding also occurred. A laparotomy was performed on *baboons* 1 and 2 on the day of bleeding and a huge ovarian cyst was discovered in each case. The ovaries were removed for histological examination. The turgescent perineum of *baboon* 3 diminished rapidly in size for 48 hours and thereafter deturgescence was more gradual. On *day* 9 after the injection perineal rest was attained and it was sustained at this level for 3 days; without the intervention of bleeding a new cycle was initiated. Bleeding occurred on the 49th day of the cycle or 41 days after the injection and the experimental cycle was prolonged (table 1). The baboon bled normally for 4 days and the perineum commenced to rise. Three days after the menstrual bleeding had stopped and when the perineum was 4 inches above the resting level bleeding recurred. A laparotomy was performed and again a cystic ovary was removed.

For the first few days after the injection *baboon* 4 responded in very much the same way as the other 3 baboons, except that the deturgescence of the perineum was much more evenly maintained (fig. 1). Ten days after the injection perineal rest was reached and bleeding occurred. In this case the cycle was shortened by 18 days and the succeeding cycle was perfectly normal.

In all 4 baboons, therefore, 5 mg. of estradiol benzoate and 20 mg. of progesterone caused the turgescent perineum to return to rest by the 9th to 10th day. In one baboon bleeding occurred at this stage (*baboon* 4). In the other three, after 4 to 5 days of rest, turgescence was renewed and in two baboons (1 and 2) bleeding occurred while the perineum was still turgescent. In *baboon* 3 an almost normal cycle followed the induced perineal rest.

Experiment 2 (5 mg. of estradiol plus 10 mg. of progesterone; *baboons* 5, 6, table 1, fig. 1). The reaction was similar to that described for *baboon* 3 except that bleeding occurred on the 56th instead of the 49th day and after the cessation of bleeding there was no recurrence of the bleeding.

Experiment 3 (5 mg. of estradiol plus 5 mg. of progesterone; *baboons* 7 to 14, table 1). Five of the 8 baboons responded very much in the same fashion. Twenty-four to forty-eight hours after the injection the perineum underwent deturgescence and reached its resting level within 7 to 9 days. The perineum remained at rest until between *days* 27 and 32 of the cycle when bleeding occurred and the cycles were shortened by 6 to 12 days (fig. 2). *Baboon* 9 reacted in a manner identical to that of *baboons* 5 and 6. In *baboon* 11, however, the turgescent perineum returned to rest on the 8th day after the injection. After 4 days of rest the perineum rapidly reached

TABLE 1. EFFECT ON CYCLE LENGTH AND ON THE PERINEUM OF BABOONS OF THE ADMINISTRATION OF VARIOUS COMBINATIONS OF ESTRADIOL BENZOATE AND PROGESTERONE AS A SINGLE INJECTION ON THE 8TH TO 10TH DAY OF THE CYCLE

Baboon no. (B)	Combined injection of		Earliest effect on perineum after injection	Duration of effect on perineum	Length of normal cycle	Length of experimental cycle
	Estradiol	Progesterone				
	mg.	*mg.*	*days*	*days*	*days*	*days*
1	5	20	2	13	39	32[1]
2	5	20	2	14	38	33[1]
3	5	20	1	12	37	49[1]
4	5	20	1	10	38	20
5	5	10	2	13	39	56
6	5	10	1	15	38	56
7	5	5	1	21	40	32[2]
8	5	5	1	18	38	27
9	5	5	3	12	40	56
10	5	3	1	23	39	33[2]
11	5	3	3	14	38	38[2]
12	5	3	2	19	38	28[2]
13	3	5	4	22	42	32[2]
14	3	5	2	24	40	33
15	2	1	2	15	39	29
16	2	1	1	21	38	31

[1] Bleeding while perineum turgescent; laparotomy performed and large cyst on 1 ovary discovered.
[2] Partial or complete perineal turgescence succeeding the perineal rest induced by combined injections.

maximum turgescence and within 6 days underwent deturgescence once more, returning to rest, and then rose sharply for 1 day when bleeding occurred on the 38th day of the cycle or 28 days after the injection. Thus this baboon exhibited two short perineal cycles, one artificially induced and the other spontaneous. Although the cycle was normal in length from the point of view of the interval elapsing between one menstruation and the next, nevertheless the perineum showed definitely that the ovaries were functioning in an abnormal manner. There were all gradations between this type of reaction exhibited by *baboon* 11 and those of *baboons* 8, 13, 14 (fig. 2). In those baboons (7, 10, 12) where the perineal rebound was partial as compared with *baboon* 11, the cycle was shortened as in *baboons* 8, 13, 14.

Thus 5 mg. of estradiol benzoate plus 5 mg. of progesterone evoked a diversity of responses some of which were similar in character to those evoked by 5 mg. of estradiol benzoate alone and others similar to those baboons treated with 5 mg. of estradiol and 10 mg. of progesterone.

Experiment 4 (*baboons* 15 and 16, table 1). These animals injected with lesser quantities of estradiol and 2 mg. of progesterone (*baboon* 15) and 1 mg. of progesterone

(*baboon 16*) reacted in the same manner as *baboons 13* and *14*. The perineum underwent deturgescence rapidly and after a rest period of 14 to 16 days bleeding occurred.

COMMENT

The reactions of the perineum after any of the combinations of estradiol and progesterone used with 3 exceptions (*baboons* 9, *11*, *13*) precipitated perineal deturgescence within 48 hours. This must be regarded as a progesterone effect (1). In the exceptional baboons perineal deturgescence set in on the 3rd to 4th day after the injection. This resembles more the estrogen type of response. It may be significant that the baboons which reacted in this fashion received 5 mg. of progesterone or less

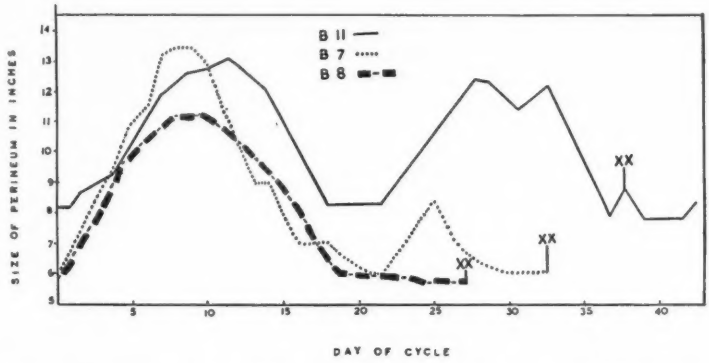

Fig. 2. Effect of various combinations of estradiol benzoate and progesterone injected in the 1st part of the cycle on the size of the perineum and on the onset of bleeding. Note the great rise of the perineum (perineal rebound) after the induced rest in *B 11*. In *B 7*, perineal rebound is less and in *B 8* it is absent. See table 1 and text for details. XX denotes onset of bleeding.

and the progesterone action in these instances may have been overshadowed by the estradiol. It seems, therefore, that the initial reactions of the perineum can be attributed largely to progesterone.

Once deturgescence of the perineum occurs after combinations of estradiol and progesterone, the perineum returns very slowly to the resting level which is attained by the 9th day. After progesterone, perineal rest is complete within 4 to 7 days following the injection, whereas after estrogen, rest is attained between the 10th and 12th days. The slow deturgescence in these experimental animals is more closely akin to the estrogen response than to progesterone. The duration of the effect elicited by the combined injections lasted for 10 to 24 days although in 9 animals it was between 10 and 15 days (table 1). Those animals that had recovered between the 10th and 15th day after the injection appear to be chiefly influenced in this respect by the estradiol rather than by progesterone. Those animals that recovered slowly fall into a different category and will be more fully discussed below.

The perineal response of the baboons injected with 5 mg. of estradiol and 20 mg. of progesterone behaved in an unexpected fashion. In all previous experiments where 20 mg. of progesterone alone was used the turgescent perineum returned very rapidly to rest and bleeding was precipitated within 7 days of the injection. Twenty mg. of progesterone combined with 5 mg. of estradiol benzoate in 3 of the 4 baboons did not produce bleeding as after 20 mg. of progesterone alone. In *baboon 4* bleeding was precipitated prematurely on the 10th day instead of between the 4th and 7th days. In *baboons 1*, *2* and *3* therefore the estradiol inhibited the bleeding action of the pro-

gesterone. In *baboon 4*, however, the bleeding can be regarded as resulting from the withdrawal of estrogen rather than from the withdrawal of progesterone. It follows that where progesterone alone is administered in sufficient amounts in the first part of the cycle the formation of estrogen by the ovaries is suppressed and progestogen withdrawal bleeding is precipitated. When estrogen is administered concurrently with progesterone, then, although the formation of estrogen by the ovaries is temporarily disturbed, the exogenous estrogen has been only partially used up and there was sufficient to overwhelm the bleeding effect of progesterone. The ovaries had recovered by the 10th day when the exogenous estrogen had been metabolized and therefore estrogen withdrawal bleeding was prevented and the perineum commenced to rise again. In *baboon 4*, although the exogenous estrogen had prevented the onset of bleeding after the progesterone had been completely metabolized the exogenous estradiol inhibited the ovaries to such an extent that when it had been completely metabolized by the 10th day, the ovaries had not as yet taken up their function and consequently estrogen withdrawal bleeding occurred. Thereafter a completely new cycle was initiated.

In *baboons 1, 2* and *3* there was no bleeding within 10 to 16 days of the combined injection, but a seemingly new cycle was initiated. This was very short in *baboons 1* and *2*, and slightly longer in *baboon 3*, but in all 3 the ovaries were so damaged that bleeding occurred while the perineum was turgescent. One of the ovaries in each of these 3 animals was cystic. This abnormal bleeding cannot be regarded as following on hyperestrogenic stimulation because had this been the case the perineum would have been swollen at least to the same extent as seen normally at the height of estrus, but instead at the time when these animals bled abnormally the perineum in *baboons 3* and *4* although turgescent was well below the maximum size normally attained (fig. 1). Bleeding thus occurred from an endometrium partially stimulated by estrogen. All that can be said in explanation is that cystic ovaries were found in each case. In *baboons 1* and *2* these were present 24 days after injection and in *baboon 3* by the 44th day. It seems that the onset of bleeding in *baboon 4* in some way regulated the succeeding cycle, and, although cystic ovaries might have developed the cycle was not disturbed. No laparotomy was performed on this animal as previous work has shown that any handling of the ovaries promptly disturbs the menstrual cycle (4).

It has been shown that 3 to 15 mg. of progesterone administered in the first part of the cycle causes perineal deturgescence and rest and then, without the intervention of bleeding, a new but slightly abbreviated perineal rhythm is initiated. The experimental cycle is lengthened. When 10 mg. of progesterone is combined with 5 mg. of estradiol (*baboons 5* and *6*) perineal deturgescence is induced and after a period of rest a new perineal cycle is initiated without the intervention of bleeding and the whole experimental cycle, of which the abbreviated one is a part, is greatly lengthened. The lengthening of the cycle is almost equivalent to that obtained after 1 to 5 mg. of estradiol alone although in the latter case the cycle is slightly longer than after 10 mg. of progesterone alone. Such a perineal response and lengthening of the menstrual cycle was also elicited by giving 5 mg. of estradiol and half the amount of progesterone (*baboon 9*, table 1). With lesser quantities of progesterone than that employed in *experiments 1* and *2* a variety of perineal reactions are evoked. One baboon (9), as just mentioned, behaved in a way indistinguishable from *baboons 5* and *6*. In five baboons (*baboons 8, 13, 14, 15, 16*) after the induced perineal deturgescence the perineum returned to rest and bleeding occurred on the 26th to 32nd day of the cycle, the cycle having been shortened by 6 to 12 days (fig. 2).

In *baboons 12, 10, 7, 11* a few days after perineal rest had been attained, the perineum attempted to undergo turgescence once more, but in only one baboon was

it very marked (*baboon 10*). In *baboon 12* it was very slight, in *baboon 10* it was a little more marked whereas in *baboon 7* it was definite. In the other baboons of this series the perineum remained in a state of rest until the premature bleeding occurred. It appears that if the ovaries fail to form estrogen within 8 to 10 days of the injection the perineum remains in a state of rest (*baboons 13–16*), whereas in others the ovary may recover partially and form sufficient estrogen to cause a partial turgescence (*baboons 12, 10, 7*) or there may be sufficient estrogen to cause maximum turgescence by the 12th day (*baboon 11*) (fig. 1). It is surprising that in *experiment 3*, with one exception (*baboon 9*), the cycle was shortened whether the ovaries did or did not commence to function by the 8th to 10th day after the combined injection of 5 mg. or less of estradiol benzoate together with 5 mg. or less of progesterone (table 1).

Thus 5 mg. or less of estradiol benzoate combined with 5 mg. or less progesterone caused early perineal deturgescence and with one exception, a shortening of the cycle. The early deturgescence was obviously due to the presence of progesterone, but the shortening of the cycle could not be attributed to the progesterone alone, nor to estradiol benzoate alone as the latter only led to premature bleeding in one of 10 baboons (*baboon 2*). The quantities of estradiol and progesterone employed in *experiments 3* and *4* apparently inhibited the ovaries to a much greater extent than in *experiments 1* and *2*.

The behavior of the perineum in this series of experiments affords significant information concerning the effect of combined injections of estradiol benzoate and progesterone. An inspection of figures 1 and 2 shows very clearly the nature of the ovarian response to such a combined injection. The extent of the ovarian inhibition is paradoxically less after injections of 5 mg. of estradiol and 10 mg. of progesterone than after 1 to 5 mg. of estradiol and 1 to 5 mg. of progesterone. From the perineal reaction it is possible to understand clearly what actually happens when the cycle is lengthened. In figure 1 for example, it is quite obvious that although the cycle is lengthened and the onset of menstruation delayed the ovaries must have been undergoing a cyclical change otherwise it would not have been possible for the perineum to have passed through the type of rhythm which is shown in that figure. Again from figure 2 it is equally obvious that the interval between bleeding was normal, and if that criterion alone were used, as is the case in studies of the menstrual cycle in women, it would not have been possible to have arrived at the conclusion that the cycle was profoundly disturbed even though the bleeding appeared almost at the normally expected time. Such studies in the baboon show clearly how difficult it is to state with any certainty that a menstrual cycle of average length is physiologically normal, particularly in women.

SUMMARY AND CONCLUSIONS

The action of combinations of estradiol benzoate and progesterone simultaneously administered have been examined using the perineum as an indicator of ovarian activity. Varying grades of ovarian inhibition have been described. Attention was drawn to the fact that cystic ovaries have been induced within 24 to 34 days in 3 of 4 animals injected with 5 mg. of estradiol benzoate and 20 mg. of progesterone.

It was shown that 5 mg. of estradiol benzoate and 20 mg. of progesterone caused the turgescent perineum to return to rest by the 9th to 10th day in 4 baboons used for this purpose. In one baboon bleeding was premature and the cycle was shortened by 18 days. In the 3 other baboons the perineum commenced to swell again after the induced deturgescence and bleeding occurred in 2 of these 3 animals on the 32nd day of the cycle while the perineum was still turgescent; in the remaining animal the cycle was prolonged by 12 days.

Five mg. of estradiol benzoate and 10 mg. of progesterone lengthened the cycle by 17 days. Smaller quantities of estradiol benzoate and progesterone elicited a variety of responses, but in general with one exception the cycle was shortened.

The deturgescence of the perineum appears to be due to the progesterone and thereafter the estrogen exerts its effects.

The bleeding induced by 20 mg. of progesterone in the first part of the cycle can be inhibited by the simultaneous administration of 5 mg. of estradiol benzoate.

Ten mg. of progesterone together with 5 mg. of estradiol benzoate produced a result very similar to that achieved with estradiol alone.

Small quantities of estradiol benzoate and progesterone caused marked ovarian inhibition which was greater than after estradiol alone. The ovaries were partially or completely inhibited.

Attention is drawn to the fact that in women a menstrual cycle of average length may not necessarily be normal physiologically.

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TEMPORARY OVARIAN DAMAGE PRODUCED IN BABOONS BY SINGLE ADMINISTRATIONS OF ESTRADIOL BENZOATE AND PROGESTERONE IN THE FIRST PART OF THE CYCLE[1]

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IT HAS BEEN SHOWN that single injections of estradiol benzoate, progesterone or of combinations of both into normal adult female baboons in the first part of the cycle will cause the turgescent perineum to undergo deturgescence (11, 12, 13). Deturgescence of the perineum is normally encountered in the second part of the cycle when the corpus luteum develops on the ovary. In castrate animals treated repeatedly with estrogen the perineum becomes turgescent, but when the estrogen is withdrawn the perineum undergoes deturgescence. It was felt that progesterone could cause perineal deturgescence by acting directly on the perineum as this substance is normally present in the second half of the cycle. Gillman and Stein (16) have shown that, even in the presence of enormous quantities of estrogen, the perineum will undergo deturgescence, provided adequate quantities of progesterone are simultaneously administered. It must be remembered, however, that when progesterone is given in the first part of the cycle, the ovaries are normally secreting estrogen and, according to the generally accepted theories, there is no progesterone present, this latter substance being formed only after ovulation. Even though the progesterone acted directly on the perineum it was worth while to discover whether the ovary was in any way affected by the presence of this exogenous progesterone at a time when it is said to be absent, especially as 20 mg. of progesterone will cause not only deturgescence, but also premature bleeding.

Again, it is generally accepted that estrogen is responsible for perineal turgescence in both normal and castrated baboons (14). When normal baboons receive estradiol benzoate as a single injection in amounts of 0.1 mg. or more in the first part of the cycle, the perineum paradoxically undergoes deturgescence and only 10 to 16 days after the injection does the perineum become turgescent once more (11). Moreover, when estrogen is repeatedly given to normal baboons in the first part of the cycle the perineal turgescence is sustained for as long as the estrogen is continued. On withdrawal the perineum soon undergoes deturgescence (14).

The object of this paper therefore, was to ascertain, as far as histological examination allowed, the state of the ovaries in the experiments in which *a*) estradiol alone, *b*) progesterone alone or *c*) combinations of both were administered as a single administration in the first part of the cycle. Mention will also be made of the state of the ovaries removed from animals which had received estradiol benzoate repeatedly over a considerable period. At the same time the behavior of the perineum in response to these hormones will be examined in relation to the changes induced in the ovaries.

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MATERIAL AND METHODS

Baboons were injected with estradiol benzoate and/or progesterone on the 8th to 10th day of the cycle. The details are stated in the protocols. The ovaries were removed as soon as the perineum reached the resting level in the case of the estrogen experiments and in experiments where small quantities of progesterone were used. In those experiments in which 20 mg. of progesterone was injected the ovaries were removed on the first day of the prematurely induced menstruation, while in those animals that bled while the perineum was turgescent the ovaries were removed on the first or second day of this abnormal bleeding.

OBSERVATIONS

The effect of estrogen. Five mg. of estradiol benzoate was injected on the 8th day of the cycle and the ovaries removed 7 days later when the perineum had undergone deturgescence but was still above the resting level.

Macroscopically the ovaries were about the same size as those removed from a normal animal on the corresponding day of the cycle. In section under low power the ovary appeared more solid than usual due to the absence from the cortex of the numerous large Graafian follicles (fig. 2). In an ovary removed on the 15th day of the cycle the cortex was crowded with large well developed follicles (fig. 1). In the estrogen-treated baboon there was atresia of almost all of the large follicles and even of the smaller ones. Almost every stage in the atretic process could be observed. In fact, in studies of follicular atresia, at least in baboons, this technic of giving estrogen in the first part of the cycle can be strongly recommended.

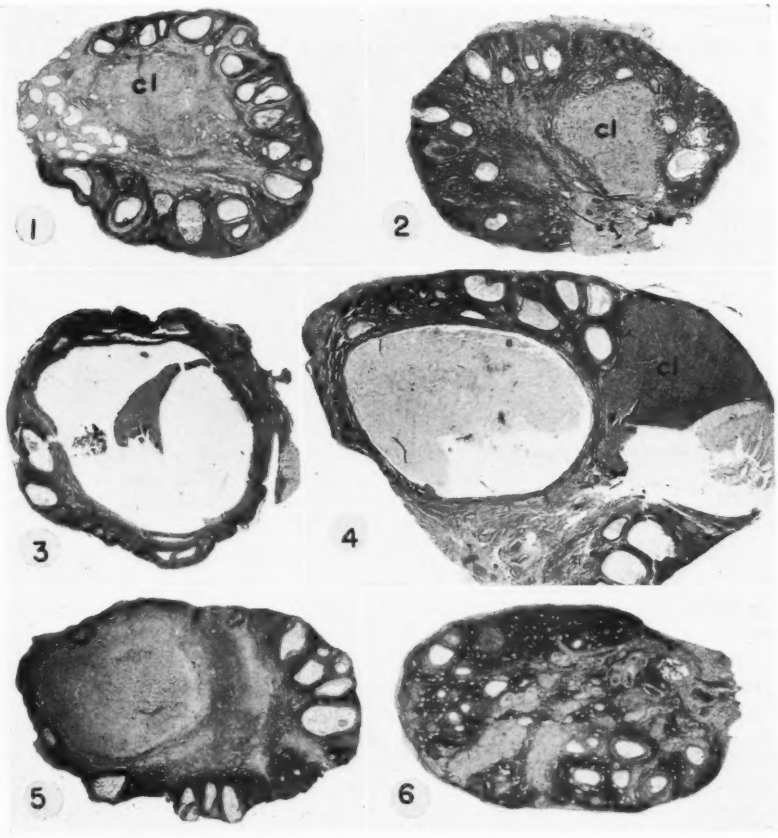
It is not the purpose of this paper to describe the atretic process, but mention is made of the main reactions in the ovary. In the large follicles there was an extensive desquamation of the stratum granulosum which also involved the cumulus oöphorus, where the ovum in some instances was so denuded of cells that it was naked and freely exposed to the follicular fluid. The desquamated granulosa cells maintained their shape for a time and imparted a granular appearance to the contents of the follicle (fig. 8); later only their outlines were visible; finally these cells liquefied and mingled imperceptibly with the follicular contents. In a large number of follicles of this type there was no reaction of the cells of the theca interna, and the theca externa as well as the surrounding stroma were intensely edematous. This perifollicular edema may be regarded as evidence of active absorption of the follicular fluid. As the follicles decreased in size the perifollicular edema subsided, the theca interna became thicker and this was associated with an ingrowth of capillaries. The layer of reticulum originally separating the stratum granulosum from the theca interna became cellular in some of those follicles which were denuded of their granular cells; some of the cells on the inner side of the theca interna were altered in their direction and came to lie at right angles to their original long axis. Later in this region fibrous tissue was laid down and this increased progressively in thickness at the expense of the cavity and also of the theca interna. Often in such atretic follicles the remains of the ovum could be discovered closely surrounded by the inner wall of dense fibrous tissue.

The cells of the theca interna appeared to assist in forming fibrous tissue, as all transitions were to be found between the fibroblast and the theca interna cells; in a well advanced stage almost all of the theca interna cells resembled fibrocytes.

The ovum usually persisted in the smaller follicles even though the atretic process was well advanced. In the very late stages of atresia, small round or oval masses were found scattered throughout the ovary. These masses consisted of spindle-shaped cells radially arranged around a small fibrous core which might or might not contain

capillaries. The spindle-shaped cells merged with the general stroma of the ovary. Estradiol benzoate therefore given as a single dose in the first part of the cycle causes extensive atresia of the large follicles and even of many of the smaller ones.

The effect of 5 mg. of progesterone. After receiving 5 mg. of progesterone on the 8th day of the cycle the perineum underwent deturgescence and when 7 days later the perineum began to swell ovariectomy was performed.



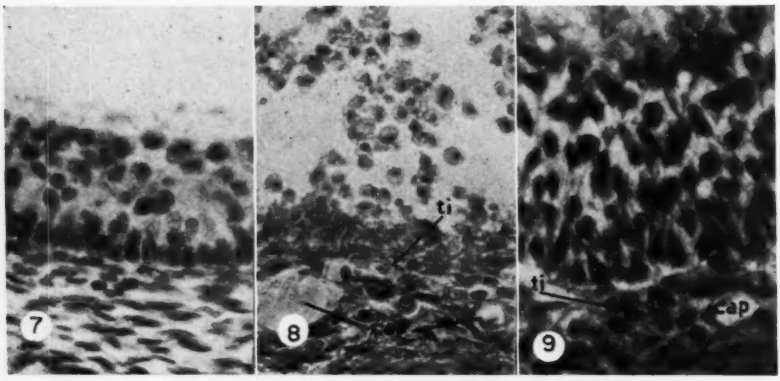
OVARIES FROM BABOONS. ×7.5. Fig. 1. Normal ovaries. c.l., old corpus luteum. Fig. 2. Ovary removed 7 days after a single injection of 5 mg. of estradiol on the 8th day of cycle. Note desquamation of the stratum granulosum in every follicle and massive atresia. c.l., old corpus luteum. Fig. 3. Ovary removed 7 days after a single injection of 5 mg. of progesterone on 8th day of cycle. Note the large cyst. Fig. 4. Ovary removed 7 days after a single injection of 20 mg. of progesterone on 8th day of cycle. Note 2 large cysts. c.l., old corpus luteum. Fig. 5. Ovary removed 24 days after a single injection of a combination of estradiol with progesterone. Note cyst with semisolid contents. Fig. 6. Ovary removed 23 days after daily injections of estradiol benzoate commencing on 8th day of cycle. Note the solid appearance of ovary with evidence of massive atresia of majority of follicles and persistence of some large follicles with thickened capsules.

Macroscopically the ovary was slightly larger than the normal. In section it was immediately apparent that almost the whole length of the ovary was occupied by a cyst measuring 6×6×10 mm. (fig. 3). This cyst was centrally situated and in the

compressed cortex a few distorted follicles were present. These follicles were much larger than in the ovary of the animal treated with 5 mg. of estradiol benzoate.

The cyst was filled with a coagulum and it was lined by a layer of granulosa cells which in places were several layers thick. In some regions these granulosa cells were histologically quite normal, whereas in other regions there was a layer stretched so thin that it was almost unrecognizable.

The majority of the follicles, although distorted, were lined by a thick, healthy stratum granulosum which consisted of several layers of cells. Some of the large and even of the medium-sized follicles showed desquamation of the stratum granulosum,



GRANULOSA TISSUE IN OVARIES OF BABOONS. ×500. Fig. 7. Stratum granulosum of fully formed normal follicle. Fig. 8. Desquamating granulosa cells in follicles of same ovary as fig. 2. *t.i.*, cells of theca interna; *cap.* dilated capillaries. Fig. 9. Wall of large cyst shown in fig. 4. Note much thickened layer of granulosa cells, enlarged cells of theca interna (*t.i.*) and dilated capillary (*cap*) in the theca interna.

but the number of follicles undergoing atresia was significantly less than after estrogen treatment. Atresia of the smaller follicles was absent.

The effect of 20 mg. of progesterone. The animal was injected with 20 mg. of progesterone on the 8th day of the cycle and 7 days later the perineum was at rest and bleeding was precipitated. The cycle was shortened by 23 days. On the 2nd day of bleeding ovariectomy was performed.

Macroscopically the ovary was at least 3 to 4 times larger than the normal. In section the ovary was seen to be occupied by 2 large cysts even larger than that observed following 5 mg. of progesterone (fig. 4). Again the cysts occupied the center of the ovary extending to the surface in one or two places. Scattered in the cortex and superficial to the cysts were a number of large follicles. This was particularly obvious in those parts of the ovary situated beyond the cyst. Under low power the cyst was lined by a stratum granulosum which was much thickened in some parts and thin in others. The cumulus oöphorus was identified despite the great tension in the cyst. The stratum granulosum was definitely modified as compared with that seen in a normal follicle. The cells were stellate and frequently assumed a form which is usually encountered in a very early corpus luteum (fig. 9). In fact these cells may be regarded as pre-luteal cells. In those parts where the stratum granulosum consisted of one layer only, the cells were spheroidal and very unlike the much flattened cells which usually line a tense cyst. The outer layers were vascular and the surrounding ovarian stroma was moderately edematous. The large follicles were lined by a thick

stratum granulosum in which numerous mitotic figures were to be seen. Many of these follicles were larger than those usually present in the ovary at the time of ovulation. Some of the large follicles showed massive desquamation of the stratum granulosum, but this feature was not nearly so common as in the ovary of the animal treated with estrogen. On the other hand the atresia of small and intermediate follicles was a notable feature. In two instances an intermediate follicle undergoing atresia showed luteinization of the granulosa cells associated with an enlargement of the cells of the theca interna. Atretic follicles were frequently seen with the ovum persisting in the center even though the process was advanced.

Combination of estradiol and progesterone. Three baboons were injected with 5 mg. of estradiol benzoate and 20 mg. of progesterone on the 8th to 10th day of the cycle. The reactions of the perineum have already been described in considerable detail (12). The ovaries were removed on the 2nd day of bleeding and at the time when the perineum was still turgescent. The ovaries were removed 24 days after injection in 2 baboons and on the 44th day in the 3rd.

The ovary removed on the 24th day following injection was not very much enlarged. In section 2 cysts were present at different levels in the ovary, one measuring 10 mm. in diameter and a smaller one whose diameter was 6 mm. The larger cyst is depicted in figure 5. Numerous follicles could be seen lying on the periphery of the ovary.

Under low power (×80) the larger cyst contained a central coagulum which stained intensely with eosin; it seemed to be semi-solid in consistency. The structure of the cyst wall varied considerably; in some parts it was lined by a cellular fibrous tissue and many of these cells could be seen extending for some distance into the coagulum. Deep to this fibrous layer were a few clusters of thecal cells, separated from each other by strands of vascularized stroma. In other parts a thin layer of granulosa cells was present while in still another part there was a thick layer of enlarged thecal cells which formed a prominent cap to the cyst. These thecal cells were so large that they could easily be mistaken for lutein cells. The margin of this layer in contact with the coagulum was fibrous tissue which also invaded the coagulum to a varying extent.

The smaller cyst was lined by a thick layer of granulosa cells. The cumulus oöphorus was identified and it contained a degenerating ovum. The extent of the cellular bed of the stratum granulosum was apparent in one ovary which had ruptured accidentally at operation.

There was evidence of extensive atresia of follicles in the large numbers of small round cellular masses which were seen to enclose central cores of fibrous masses of irregular size. In some of the larger follicles in which the atresia had not proceeded to this extent the stratum granulosum had desquamated and the cells were scattered throughout the follicular fluid.

Prolonged treatment with estradiol benzoate. A normal baboon was injected daily with 1 mg. of estradiol benzoate for 23 days commencing on the 8th day of the cycle. The perineum was maintained in a state of maximum turgescence and on the 31st day of the cycle both ovaries were removed while the perineum was turgescent.

The ovaries were larger than normal, but only a very few follicles could be identified with the naked eye (fig. 6). Under low power the stroma had a blotchy appearance especially in the superficial part of the cortex. This blotchiness was due largely to the breaking up of the stroma into a number of discrete areas which were obviously the end phases of atretic follicles; in some the fibrous centers could still be distinguished. Even the very small follicles, surrounded by only a few layers of granulosa cells, were undergoing degeneration.

Only a very few of the medium-sized follicles appeared to be normal. In these the

theca interna was relatively thick and certainly very much thicker than is encountered in healthy follicles of the same size, but the stratum granulosum was desquamated. There was no recent corpus luteum on the ovary and it was clear that not only had ovulation been inhibited, but also that there had been a gross interference with the ripening of follicles. In addition there was evidence that the large numbers of follicles present in the ovaries before treatment with estradiol had become atretic. No follicular cysts were found in the ovary.

DISCUSSION

The administration of a single dose of 5 mg. of estradiol benzoate causes massive atresia of the follicles of the ovary and no corpus luteum is formed. The perineal deturgescence which is known to follow within 4 to 11 days of such a treatment is caused by the absence of estrogen. This single administration of sufficient estrogen, therefore, will inhibit the production of endogenous estrogen for 10 to 16 days. Thereafter the ovaries commence to produce estrogen once more and this is associated with a renewed turgescence of the perineum.

A single administration of estradiol to baboons in the first part of the cycle causes massive follicular atresia. In 40 to 50 gm. rats Hohlweg (17) claims to have induced corpus luteum formation by the administration of a single large dose of estrogen. Desclin (7) confirmed this observation but Bachman (1) failed to duplicate Hohlweg's experiments. In rabbits moreover, Bachman did not observe any change in the minute anatomy of the ovaries 48 hours after a single injection of a large amount of estrone. It is evident, therefore, that in response to a single injection of estrogen the ovary of the baboon responds differently from that of rats and rabbits. Continuous daily treatment with 1 mg. of estradiol benzoate commencing early in the first part of the cycle in the baboon leads to the disappearance of the larger and medium-sized follicles. Zondek (21) recorded the atrophy of the ovaries of a woman who had received massive quantities of estrogen. Unfortunately in his experiment, the woman had cancer and there were secondary nodules in the ovary. Nevertheless the experiments in baboons seem to lend support to his observations that massive treatment with estrogen causes profound ovarian inhibition. This is a matter which must receive serious attention in view of the fact that estrogens in much smaller doses than are used clinically can cause ovarian inhibition. While normal women apparently recover rapidly, treatment with estrogen in those women whose ovaries are not functioning actively may result in a prolonged period of amenorrhea in which there is no detectable ovarian activity. In such cases estrogen therapy may do much more harm than good.

The repeated administration of estrogen to baboons in the first part of the cycle causes the perineal turgescence to be sustained for as long as the estrogen is given. Thereafter the perineum undergoes deturgescence. The perineal turgescence in this instance is obviously due to the exogenous estrogen acting on the perineum as the ovaries are very profoundly inhibited. Mention was made of the fact that in the ovary treated with estrogen for 23 days there were a few medium-sized follicles present. This suggests that baboons might develop some resistance to estrogen in the same manner as reported by Selye (20) for the rat. In baboons, however, this matter requires further investigation.

Whereas a single treatment with estrogen causes wholesale atresia of the follicles with a diminution in estrogen production by the ovaries and a consequent premature deturgescence of the turgescent perineum, progesterone acts in an entirely different way. Gillman and Stein have shown that in castrated baboons previously treated with estrogen the artificially induced turgescence of the perineum can be made to undergo

deturgescence by supplying adequate quantities of progesterone. In this case it seems that the progesterone acts directly on the sexual skin and causes a rapid transference of water from these tissues. Hence the sexual swelling diminishes rapidly in size.

In normal baboons on the 8th to 10th day of the cycle there is an active formation of estrogen, so much so that the perineum enlarges very rapidly (several inches in 24 hours). The administration of progesterone, at this stage, apparently has a similar action on the turgescent perineum as in a castrate and hence deturgescence supervenes; within 5 to 7 days the progesterone is metabolized and turgescence is initiated once more. From an examination of the curves of the perineum this was the obvious explanation of the perineal response to progesterone whether bleeding was induced or not. It was not suspected in the early experiments (12) that any injury was inflicted on the ovaries. The discovery that progesterone leads to the formation of a cyst on the ovary within 5 to 7 days was surprising. Cystic ovaries have been produced in this fashion in all 7 animals thus far examined. So constant is this reaction that in baboons it can be reliably recommended as a technic for producing ovarian cysts and for studying their development and involution. These cysts form very rapidly. They are follicular cysts as, in almost all cases, examination of serial sections has resulted in the identification of the cumulus oöphorus. Histologically the stratum granulosum of such cysts is extremely active (fig. 9); in the cyst ruptured at operation there was an enormous bed of active cells. In addition to the development of the cyst, progesterone also caused slight atresia of the follicles, but this was not nearly so extensive as in cases where estradiol alone was used. That relatively few follicles had been inactivated by the progesterone is also reflected in the rate of recovery of the perineum, in which within 5 to 7 days turgescence was evident once more. On the contrary, after estradiol, 10 to 16 days must elapse before turgescence is renewed.

It is quite clear from the foregoing that progesterone in the first part of the cycle acts not only on the perineum but also causes profound change in the ovaries as witnessed by the development of a large follicular cyst.

Combinations of estradiol and relatively small quantities of progesterone cause cystic ovaries and massive atresia of the follicles. For this reason the perineum undergoes deturgescence much more rapidly after the progesterone than after estradiol alone; perineal rest is sustained for a longer period than after progesterone alone. The rapid recovery of the perineum seen after progesterone alone is prevented by the addition of the estradiol, as the follicles are more extensively damaged and recovery is much slower. Even so, the combinations of estradiol and progesterone seem to inflict greater damage on the ovaries than can be accounted for by the arithmetical effects of these 2 hormones. This is especially well shown by the behavior of the perineum as already described (11). Also the menstrual rhythm is so affected by a combination of 5 mg. of estradiol and 20 mg. of progesterone that in 3 of 4 baboons, the perineum was almost maximally turgescent and bleeding occurred. The association of turgescence with bleeding is a combination of events which has never been encountered so far in normal animals. The presence of the cyst does not necessarily mean that it was directly responsible for the bleeding as 5 mg. of progesterone alone also caused the formation of a cyst, but in this case was unattended by bleeding. The endometrium in such cases looks very much like that usually encountered in the first part of the cycle. The etiology of the bleeding is unknown, but it cannot be regarded as being caused by an excessive amount of estrogen as the perineum at the time of this abnormal bleeding had not attained maximum turgescence such as seen normally in those animals near ovulation. Further study of this type of bleeding should afford important information concerning the problem of abnormal bleeding in women.

Combinations of estradiol benzoate and progesterone therefore lead to marked

ovarian inhibition associated with the development of a cyst on the ovary. A combination of 5 mg. of estradiol benzoate and 20 mg. of progesterone, profoundly disturbs the menstrual cycle temporarily and is associated with an abnormal type of bleeding.

It is not possible at this stage to explain precisely the mechanism involved in the production of follicular atresia by estradiol or the development of the follicular cyst after progesterone. An examination of some of the endocrine glands in animals treated with their own specific hormones, however, might afford some information in this connection. Thyroxin administered to normal animals causes the thyroid follicle to increase in size. When the supply of exogenous thyroxin is suspended the follicle diminishes in size, due to the absorption of the stored secretion. Again, the administration of a single injection of parathormone leads to a diminution of the volume of the cell with retraction of the cytoplasm and hyperchromasy of the nucleus of the cells of the parathyroid gland. Hydropic degeneration of the cells may also occur and this is sometimes associated with the accumulation of a colloid substance (6). Similarly Gersh and Grollman (9) have shown that in immature mice the administration of small doses of cortical hormone leads to the disappearance of the x zone of the suprarenal gland. These investigators are of the opinion that the x zone acts as a reserve tissue which can be depressed when the need for cortical hormone is satisfied in some other way.

In each of these 3 endocrine glands it follows that the administration of hormone is followed by specific histological change in the gland concerned. By analogy, it seems that the degeneration of the stratum granulosum of the large and small Graafian follicles was due to the exogenous estrogen which was present in much greater concentration than required by the body at that particular moment in time and consequently the estrogen-producing cells were inhibited. This inhibition expressed itself in the form of a degeneration and desquamation of the granulosa cells which can be regarded as the first stage of follicular atresia. It is common knowledge that the Graafian follicle may terminate its development in one of 3 ways, namely, by undergoing atresia (the fate shared by the majority of follicles), by becoming a corpus luteum (the fate shared by a limited number of follicles) and finally by becoming cystic (not an uncommon phenomenon).

The administration of estrogen therefore terminates the life of the follicle by causing atresia. This is no doubt an extreme process and is greatly facilitated by the fact that the life of the Graafian follicle is relatively short as compared with that of the thyroid follicle. But even in the latter case degeneration of the epithelium can also be excited acutely by rapidly increasing the temperature of the body (8). The factors responsible for ovulation and corpus luteum formation in primates are imperfectly understood. The formation of a follicular cyst, however, indicates that the stimulus for the formation of liquor is operative in one or two follicles only but that absorption is proceeding more slowly, if at all. Progesterone, apparently, does not significantly interfere with the formation of follicular fluid, but it does interfere with its absorption. Why only one, and at the most two, follicles become cystic after progesterone is indeed a mystery, but would it be unreasonable to presume that the follicle which would normally have ruptured at ovulation is the one which has become cystic?

Extracts of anterior pituitary or anterior pituitary-like substances may cause the development of a large number of follicular cysts in the ovary (18), whereas progesterone leads to the appearance of only one and at the most two cysts. This may be of some importance in understanding the factors responsible for cystic ovaries in the human female. Progesterone either of ovarian or extra-ovarian origin present in sufficient quantities in the first part of the cycle will lead to the development of one or two large follicular cysts in the ovary of the baboon. Since the human female responds to

progesterone in the first part of the cycle in a manner very similar to the baboon (15), it would appear that in those women whose cycles were disturbed by progesterone there is every likelihood that an ovarian cyst had been produced. Fortunately, the studies on baboons reveal that this cyst involutes rapidly and ultimately disappears from the ovary without further disturbing the menstrual rhythm.

In the ovary treated with 20 mg. of progesterone alone, two follicles had undergone extensive luteinization. Massive luteinization of follicles during pregnancy in the human female has been described by Gillman and Stein (16). Black, Heyns and Gillman (3) drew attention to the presence of these luteinized bodies in association with a persistent functional corpus luteum. The author has frequently seen such structures in ovaries with a thickened tunica albuginea and associated with the presence of large cysts. Dempsey (5) found that in the gibbon, unlike other known primates, thecal luteinization was not associated with pregnancy. He was of the opinion that in the gibbon the factor responsible for thecal luteinization was elaborated in some organ other than the placenta. Since two luteinized follicles were seen in the baboon treated with progesterone, it follows that in the baboon also this phenomenon may be encountered in non-pregnant animals. Whether this process in the baboon was initiated by progresterone is open to question.

Another feature of importance emerging from these experiments is the postponement of ovulation by giving a single injection of estradiol in the first part of the cycle. Ball and Hartman (2) were the first to show that repeated injections of small quantities of estradiol in the first part of the cycle significantly delayed ovulation in the Macacus rhesus. In baboons ovulation can be delayed by giving only one injection of estradiol. It has already been shown that a single administration of estradiol on the 8th to 10th day of the cycle causes the turgescent perineum to undergo deturgescence and it is only 10 to 16 days later that the perineum becomes turgescent once more. The follicles degenerate and no corpus luteum is formed. In such an experiment the perineum becomes maximally turgescent again on the 25th day, whereas normally this would have happened about the 12th day. In normal baboons ovulation occurs approximately between the 16th to 22nd day depending on the length of the cycle, whereas after a single treatment with estrogen ovulation is delayed for at least 13 to 16 days; there is at least a period of 35 days free from ovulation.

From preliminary experiments with estradiol in women it appears that ovulation is most probably postponed in the same way as in baboons, but in the former on account of the shorter cycle this postponement is less than in baboons.

SUMMARY

A single administration of 5 mg. of estradiol benzoate in the first part of the cycle in baboons was shown to cause massive atresia of the Graafian follicles. The perineal deturgescence which follows such treatment with estradiol is due to the withdrawal of estrogen. This inhibition of the ovary lasts for 10 to 16 days, as assessed by the behavior of the perineal swelling.

Multiple injections of estradiol in the first part of the cycle cause the disappearance of almost all the large and small Graafian follicles from the ovary. The perineum is maintained in a state of turgescence throughout due to the exogenous estrogen.

A single injection of 5 to 20 mg. of progesterone on the 8th to 10th day of the cycle is followed by rapid deturgescence of the perineum. The ovaries develop one and at the most two large follicular cysts in which the cumulus öophorus can be identified. There is an atresia of some of the larger follicles. The deturgescence of the perineum is regarded as being due to a direct action of progesterone on the sexual swelling.

Combinations of estradiol benzoate and progesterone given in the first part of the cycle cause massive atresia as well as cystic ovaries. Such treatment was associated in 3 of 4 baboons with bleeding while the perineum was in a state of almost maximal turgescence. This bleeding is definitely abnormal.

It is suggested that follicular atresia following a single treatment with sufficient estradiol benzoate is due to the fact that the exogenous estrogen exceeds the estrogen produced by the stratum granulosum which therefore is first inhibited and later degenerates.

It has been shown that estradiol benzoate given in the first part of the cycle is a useful technic for inducing follicular atresia, whereas progesterone consistently induces cysts on the ovaries.

The production of cystic ovaries in the baboon by progesterone affords a new clue to our understanding of the etiology of cystic ovaries in the human female.

A single administration of estradiol benzoate in the first part of the cycle in baboons postpones ovulation for at least 35 days. It is suggested that ovulation in the human female may be postponed in the same manner as in the baboon, but for a shorter period due to the fact that the cycle of the human female is normally much shorter than that of the baboon.

I wish to express my indebtedness to Professor R. A. Dart for his advice and encouragement and to Miss Christine Gilbert for her invaluable technical assistance.

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THE RÔLE OF PROGESTERONE AND LOCAL TRAUMA IN THE PRODUCTION OF CYSTIC-GLANDULAR CHANGES IN THE ENDOMETRIUM AND HYPERTROPHY OF THE MYOMETRIUM

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IT IS WELL KNOWN that compounds possessing folliculoid or estrogenic activity may produce cystic-glandular transformation of the endometrium in intact or spayed experimental animals (1–5). This transformation, which is frequently referred to in clinical medicine as the Swiss cheese hyperplasia, is often seen in patients at a time when the clinical symptomatology and urinary hormone assays suggest that there is an increased production of folliculoid hormones. These findings make it very probable that in the majority of the cases the cause of the condition is an excessive activity of the ovarian follicles. Randall and Herrell (6) emphasized that the corpus luteum may also play a rôle in the formation of a cystic endometrium. They examined a large number of endometrial specimens removed with a biopsy curette and found that such a transformation may occur in all phases of the menstrual cycle. Indeed they concluded that "this cystic change is believed to be the result of failure or deficiency of the function of the corpus luteum."

In the present communication we wish to describe experiments in which a typical cystic-glandular transformation of traumatized areas of the endometrium was produced in spayed rats receiving very large doses of progesterone. We deemed these observations worth recording since clinicians have been using corpus luteum hormone preparations as a therapeutic measure in patients suffering from cystic-glandular hyperplasia (7).

EXPERIMENTAL PROCEDURE

Sixteen albino rats weighing 55 to 60 gm. were ovariectomized. Between the 2nd and 8th day after this operation, they were sensitized by daily injections of 20γ of α-estradiol administered subcutaneously in 0.2 cc. of peanut oil administered in 2 doses. Beginning on the day following this preliminary sensitization, they received 15 mg. of progesterone daily in 2 subcutaneous injections, each dose being dissolved in 0.1 cc. of peanut oil. On the 4th day of progesterone treatment in 8 of these animals, the endometrium of the right uterine horn was traumatized by scratching it with a surgical needle and placing a silk thread through the lumen of the organ. On the 21st day of progesterone treatment, that is, 15 days after the trauma both the traumatized and the untraumatized animals were killed.

At autopsy, it was observed that at the site of trauma, the uterus was greatly enlarged and of hard consistency while the untouched horn in the first group and both horns in the second (non-traumatized) group, merely showed the usual hyperemia and distention which follows the administration of large doses of progesterone.

Histological examination revealed that in all cases at the site of the trauma the

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endometrium had assumed the cystic glandular form. The cystic dilatation of the glands was responsible for the major part of the increase in the total width of the organ but the muscular coats were also much more developed in the traumatized than in the untraumatized regions mainly as a result of hypertrophy of the individual muscle cells. This latter observation appears to indicate that progesterone exerts a particularly pronounced stimulating effect on uterine muscle fibers if these are stretched as was the case in the area of endometrial cyst formation. It is possible that the combined effect of stretching and progesterone are responsible for the myometrial development during pregnancy. The failure of previous investigators to obtain this effect (8) is probably due to the fact that much smaller doses of progesterone were

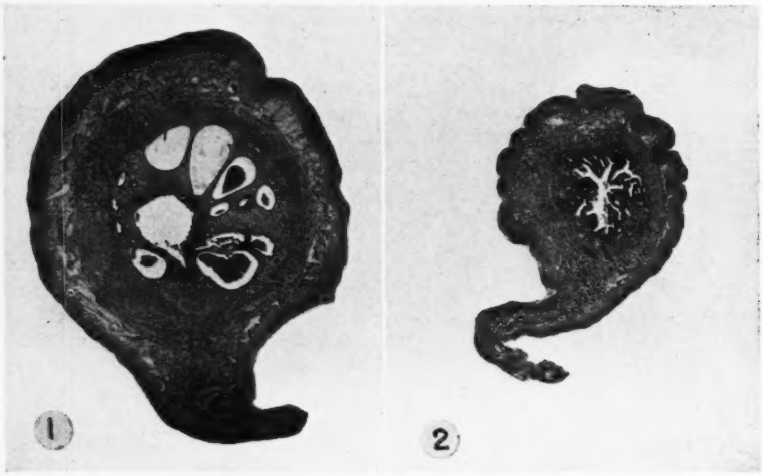

Fig. 1. TRANSVERSE SECTION THROUGH TRAUMATIZED HORN OF UTERUS OF A PROGESTERONE-TREATED RAT. Note marked cystic-glandular hyperplasia of the endometrium and increase in the thickness of the muscular coats.

Fig. 2. TRANSVERSE SECTION THROUGH UNTRAUMATIZED HORN OF UTERUS OF THE RAT SHOWN IN FIGURE 1. Note that the endometrium reveals only normal progestational changes and the myometrium is much thinner than in the traumatized area.

used. The untraumatized parts of the uterus in the first group showed marked progestational proliferation characterized by the lace-like transformation of the endometrium. In the second group in which neither of the uterne horns had been traumatized, the endometrium showed this same aspect (fig. 1 and 2).

It is noteworthy that under these experimental conditions, the traumatized regions of the endometrium showed neither deciduoma nor metrial gland formation. It may be taken for granted that during the first few days after the trauma the endometrium had been transformed into a deciduoma since this was invariably the case in a previous experimental series (11) performed under identical conditions. In these earlier experiments, we were able to show furthermore, that even if progesterone is administered daily the deciduoma involutes after a few days although the metrial gland which is formed just underneath the deciduoma in the mesometrial wall of the uterus is maintained for some time by the continued progesterone injections. We believe that in the present experimental series, the absence of deciduomata and metrial glands indicates that the large doses of progesterone were unable to maintain such

structures indefinitely. If treatment is continued as long as in the present experiments, cystic transformation of the endometrium develops in the traumatized area.

The fact that trauma played a part in the production of the cystic changes is obvious since cyst formation was seen only in the traumatized area. It remains to be seen however, whether progesterone acts as such or whether the change observed is due to phenomena caused by the gradual adaptation which animals acquire when constantly treated with this hormone. It has been shown (12) that rats chronically treated with progesterone become resistant to its effects and it is possible that the cystic changes observed after chronic treatment with this hormone are more in the nature of a 'withdrawal effect' due to a gradual decrease in the effectiveness of the hormone. It is perhaps not too far fetched to consider this possibility since uterine bleeding following a period of progesterone administration has definitely been proven to be caused by hormone withdrawal.

As an incidental observation we noted that the mammary glands of all animals showed full development such as is usually found at the end of gestation. This development must be ascribed to the action of progesterone and not to that of the estradiol administered during the pretreatment period since the breasts of four animals examined at the beginning of progesterone treatment showed no detectable signs of proliferation. This observation confirms previous reports (9, 10) according to which progesterone is capable of stimulating the growth of the mammary tissue in the rat even if no folliculoid substance is simultaneously given.

SUMMARY

Experiments in ovariectomized rats pretreated with α-estradiol and then chronically treated with large doses of progesterone have shown that if the endometrium of such animals is traumatized, it is gradually transformed into a typical Swiss cheese hyperplasia as the result of cystic dilatation of its glands. This change is preceded by the well known process of deciduoma formation at the site of trauma. The possible pathogenesis of this cystic-glandular transformation is discussed.

It has also been noted that the myometrium is much thicker in the areas distended by cyst formation than it is elsewhere. This is mainly due to hypertrophy of the individual muscle fibers. It appears that the stimulating action exerted by progesterone on uterine muscle cells is increased by the mechanical factor of distention. Attention is called to the possible implications of these observations as regards the hypertrophy of the myometrium during gestation.

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DIFFERENCE IN RESPONSE OF MICE OF DIFFERENT STRAINS TO HUMAN PREGNANCY URINE

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TESTS WERE MADE ON MICE of several strains to determine if there is a strain difference in response to pregnancy urine. Aside from being of value in eliminating strains unfitted for use in the Aschheim-Zondek test and determining particularly sensitive strains, differences in response would indicate differences in hormone balance in the strains.

PROCEDURE

Immature female mice of 6 to 19 gm. weight were used. Samples of known positive human pregnancy urine were pooled and in a 1st series of tests 1 cc. was injected subcutaneously every 12 hours until 3 cc. had been injected. The animals were examined at 12 hour intervals for opening of the vagina. They were killed 72 or 100 hours after the first injection. Stimulation of the uterus and the number of hemorrhagic follicles observed under the dissecting microscope were noted. In a 2nd series the PU factor was precipitated with 95% ethyl alcohol, washed with ether, dried and dissolved in distilled water. Injections of single doses equivalent to 3 cc. of urine were made subcutaneously. Control animals were injected in the same way with an equal amount of distilled water. Examinations were made as for the 1st series with the exception that some examinations and autopsies were made 120–123 hours after injection. In a few cases the equivalents of 6 or 9 cc. of pregnancy urine were injected.

A test was made on 2 strains of mice, one having a low and one a high response, in order to determine the earliest time after injection at which the response was evident. Examinations were made on 1 animal of each strain at 6 hour intervals after the injection of the equivalent of 3 cc. of urine.

The response of adult breeding female mice of 2 strains was tested to see if the differences were the same as in immature females. According to Sar-Louis (1) adult mice are suitable for use in the Aschheim-Zondek pregnancy test. Extract in distilled water in the equivalent of 3 or 6 cc. of urine was injected subcutaneously and examinations were made 100 or 120 hours later. Distilled water was injected in controls.

RESULTS

In the 1st series in which pregnancy urine and immature female mice were used, the vagina in all of the mice was open 12 to 72 hours after the first injection. Stimulation of the uterus bore no relationship to the presence of follicles. Mice of the A, the *C57black*, the *Swiss* and *C3H strains* had follicles but no apparent uterine stimulation. Consequently the number of hemorrhagic follicles was used as the only

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criterion. The results are shown in table 1, part 1. The *strain* A mice responded far less actively than did any of the others, averaging 0.62 follicles per ovary, as compared with an average of 3.6 for all of the others.

In the 2nd series in which a single dose of extract equivalent to 3 cc. of urine and immature female mice were used, less response in all strains was noted indicating that there was a loss of some of the activity of the PU by this method of extraction. The vagina did not open in all cases. Two *C57brown* mice examined after 100 hours showed 12 and 9 follicles respectively with the vagina not open. In one *C3H* mouse

TABLE 1. OVARIAN RESPONSE TO HUMAN PU GONADOTROPIN IN VARIOUS STRAINS OF MICE

Strain	No.	% having follicles	Av. follicles /ovary	no.	% having follicles	Av. follicles /ovary
	WT. 6–19 GM. EXAMINATION 72–123 HR. AFTER 1ST INJECTION					
	1. *Pregnancy urine, 3 cc.*			2. *Extract equivalent to 3 cc. of urine*		
A	4	75	0.62	22	18	0.09
Swiss	4	100	2.75	4	75	0.75
B.alb.C.	3	100	4.66	6	50	0.83
dba	5	100	1.30	6	100	1.16
C3H	2	100	3.00	8	100	1.68
C57black	3	100	1.66	7	86	1.71
C[e]				2	100	2.25
C57brown	4	100	5.62	10	100	3.95
Dunn's 101	4	100	3.25			
Y	4	100	3.87			
C57leaden	4	100	6.62			
	37		3.3	65		1.3
				3. *Extract equivalent to 6 cc. urine*		
A				8	50	0.56
Swiss				4	75	1.12
C57brown				1	100	4.50
				13		1.04
				4. *Extract equivalent to 9 cc. urine*		
A				2	100	0.75
C57brown				1	100	8.50
				3		6.6
	ADULT FEMALES, WT. 24–41 GM. EXAMINATION 100–120 HR. AFTER 1ST INJECTION					
	5. *Extract equivalent to 3 cc. of urine*			6. *Extract equivalent to 6 cc. of urine*		
A	5	40	1.70	2	50	1.25
C57black	6	83	3.83			
	11		2.8			

with 5 follicles the vagina had not opened at 76 hours; the vagina of 1 *dba* mouse with 4 follicles had not opened in 99 hours. The amount of uterine stimulation was rejected as a criterion in this series also. The results are shown in part 2 of table 1. Again the *A strain* of mice showed less individual response and fewer animals responding than did the others. The A mice averaged 0.09 follicles per ovary while the average for all of the other strains was 1.8 follicles per ovary. In 5 control animals receiving injections of distilled water there was opening of the vagina in 4 cases with, however, no uterine stimulation and no hemorrhagic follicles.

When the dose of PU was increased to the equivalent of 6 or 9 cc. of urine, the *A mice* responded as to 3 cc. of pregnancy urine. This is shown in table 1, part 3 and 4.

Immature *A* and *C57brown mice* were used to determine if there was a difference in the time of the appearance of hemorrhagic follicles in the ovaries. The earliest time after injection at which the follicles were found was the same for both strains.

One A and 1 C57*brown mouse* had follicles 54 hours after injection, the A *mouse* having 1 and the C57*brown* mouse, two. In neither case was the vagina open.

The response of adult breeding females of the A and C57*black strains* was compared with that of immature animals of the same strains. In both strains there was more response than in immature females, the A *animals* however increasing much more (part 5 & 6 of table 1). A much higher percentage of the tested animals responded in the adult A *mice* than in the immature animals while about the same percentage of adult and immature C57*black* animals responded. No control animal (three A and 2 C57*black mice*) showed hemorrhagic follicles.

CONCLUSIONS AND SUMMARY

There is a strain difference in the response of mice to extracts of human pregnancy urine. The A *strain* showed considerably less and the C57*brown* and C57*leaden* considerably more response than the other mice. That the difference is not due to the time of examination was shown by the fact that follicles appeared as early as 54 hours after injection in the A as well as in the C57*brown mice*.

The difference in response indicates a difference in the hormone balance in the strains of mice. That the hormone balance is changed by pregnancy and lactation is indicated by the increased response shown by adult breeding females. That the change is greater in A mice than in C57*black mice* is apparent not only from the greater increase in response but also from the increased percentage of mice responding.

The A *strain* of mice are not suited for the Aschheim-Zondek pregnancy test.

Adult breeding females of the C57*black* and A *strains* responded to human pregnancy urine as did the immature females, by formation of hemorrhagic follicles in the ovary. In no case could the appearance of the ovary of a control animal be confused with that of an ovary which had responded to PU.

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PUBERTAL INCREASE IN RESPONSIVENESS TO ANDROGEN IN THE MALE RAT[1]

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IT IS ALMOST a universally accepted belief that the individual at puberty undergoes the morphologic, physiologic and psychic transformations into the sexually mature state primarily, if not solely, as the result of marked increase in the production of sex hormones. There can be no doubt that many of the changes are due to the action of sex hormones, since they do not occur in the absence of these hormones and since they may be induced precociously by the administration of these substances.

Curiously enough, however, there are no direct data which support the notion of a significant change in the level of production of sex hormones at this period. Indeed, extracts of the testes of each of a series of bulls ranging in age from 1 month to 15 years revealed no conspicuous change in total androgen content of the testes immediately preceding, accompanying, or immediately following the onset of complete spermatogenesis and the appearance of definite signs of masculinization (1). Since castration changes are seen in rats almost as promptly following hypophysectomy (2) as following castration (3, 4) it is quite probable that the androgen content of the testes reflects the level of production of this substance. Hence it appears that these animals were undergoing pubertal changes without any appreciable change in the level of stimulating androgen. It was suggested, therefore, that at puberty the tissues had acquired the capacity to respond to levels of androgen that had previously been ineffective, that is, the tissues increase greatly at puberty in their responsiveness to androgens.

Among laboratory animals the postnatal history and control of the male reproductive system of the rat have been most thoroughly studied. Price (5) found that in early life the epithelium of the seminal vesicles is essentially like that of the adult rat showing complete castration atrophy. At the age of 36 days this epithelium has begun its transformation into the type found in the normal adult male. Thus, if the seminal vesicle is used as a test object, the rat provides a means of examining the hypothesis of pubertal increase in responsiveness to androgen. Accordingly, the experiment reported here consisted of the determination of the minimum effective dose of androgen upon the seminal vesicle of the rat at various ages.

MATERIALS AND METHODS

Since it is presumably the compound secreted by the testis and is active in the body, testosterone was the androgen selected. To provide circumstances as nearly comparable as possible 128 test animals were castrated at birth according to the

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method described by Pfeiffer (6). In groups of such castrated animals aged 10, 20, 30, 40, 50, 60, 80, 100 and 120 days, treatment was begun to determine the minimum effective dose of testosterone. To members of each group different amounts of the hormone in increments of 5 micrograms covering the range from 0 to 50μg., and occasionally higher, were administered. These amounts are absolute and have no relation to body weight. Beginning on the day in question 5 daily subcutaneous injections of the androgen were made and the animals were autopsied on the 6th day. The site of injection was varied each day to facilitate absorption. The testosterone was dissolved in sesame oil in such concentration that the daily dose was contained in 0.025 to 0.1cc. of oil.

At autopsy the animals were weighed and tissue which might possibly represent a testicular fragment was removed and sectioned serially. Testicular tisssue was found in 7 of the animals which were then excluded leaving 121 valid test animals. The accessory glands of reproduction (the seminal vesicles, coagulating glands and prostate glands, dorsal and ventral) were removed together with a small portion of urethra and weighed. These organs were fixed in Bouin's fluid, embedded in paraffin, sectioned at 8μ and stained with Ehrlich's hematoxylin with or without counter-staining with triosin.

To ascertain the sequence of events in the local colony, members of a group of intact, untreated male rats were also killed at 10, 20, 30, 40, 50, 60, 80 and 100 days of age. At autopsy, body weight, weight of the testes, of the accessories, and of the seminal vesicles alone with their contained secretion were recorded. The testes, seminal vesicles and ventral prostate glands were fixed and stained as for the operated group of animals.

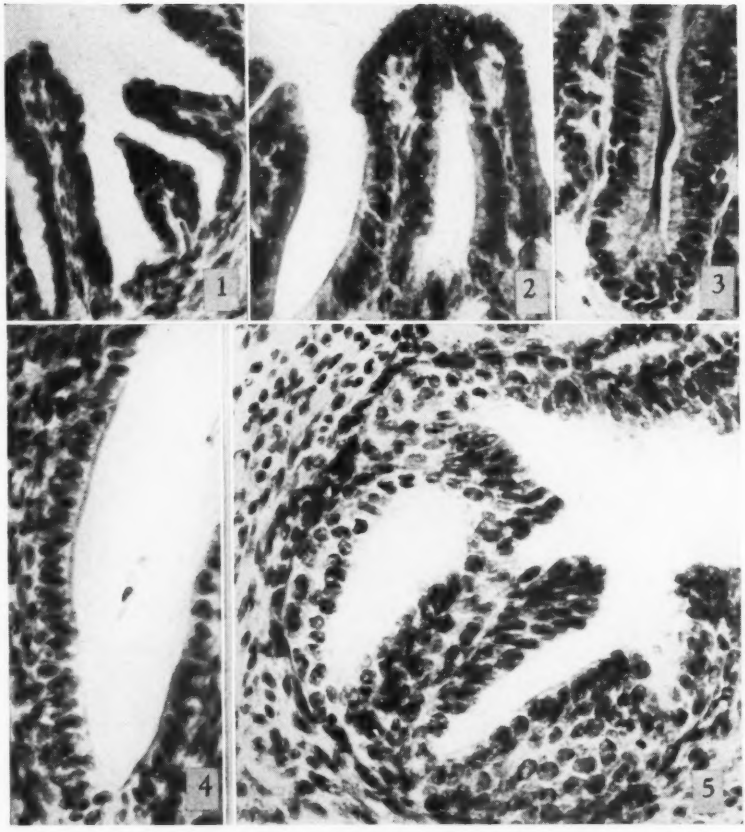
OBSERVATIONS

On the basis of spermatogenesis the onset of puberty in this colony of rats was at 40 days of age when spermatozoa were first observed in the testes, in small numbers and in only occasional tubules. The weight of the seminal vesicles alone and of the total accessories increased at a gradual and uniform rate during the period from 10 to 40 days. From 40 to 60 days the rate of growth increased markedly (fig. 6), and rapid increase in weight continued throughout the period studied.

Histologically the seminal vesicles at 10, 20 and 30 days (fig. 1) had a low, simple epithelium virtually devoid of secretion granules, and the lumina contained no secretion. The histologic picture at these ages is thus much the same as that seen several weeks after castration in the adult rat. At 40 days the epithelium of the seminal vesicles exhibited the first signs of the action of androgen (fig. 2); it was recognizably taller, contained definite secretion granules, and mitoses were numerous. Secretion first appeared in the lumen at 40 days. At 50 days (fig. 3) the epithelium was columnar, secretion granules were abundant, the lumen contained greater amounts of secretion and mitoses were very numerous. Thereafter the histological change consisted chiefly in accumulation of secretion and its distention of the entire organ. Mitoses diminished rapidly in number after 50 days.

Thus at approximately 40 days of age the seminal vesicles, grossly and microscopically, began their transformation from the infantile into the sexually mature state. The four days' discrepancy between these animals and Price's (5) rats is doubtless an inherent difference in the two stocks. On the basis of spermatogenesis and of evidence of action of androgen, puberty begins in this group of rats at approximately 40 days of age.

As found by Price (5), the prostate glands in the normal animals at all the ages studied possessed a secretory type of epithelium comparable to that of the intact



Figs. 1–5. Sections of seminal vesicles of rats. Fig. 1. Intact rat 30 days of age. The epithelium is low and virtually devoid of secretion granules. At 10 and 20 days the histology is much the same. ×180. Fig. 2. Intact rat 40 days of age. The epithelium is appreciably taller and contains secretion granules. A small amount of secretion was present in the lumen. ×180. Fig. 3. Intact rat 50 days of age. The epithelium is columnar and contains abundant secretion granules. Accumulated secretion is present between the epithelial folds. This is essentially the adult histology. ×180. Fig. 4. Rat 35 days of age but castrated at birth. The epithelium is low, simple cuboid, ×365. Fig. 5. Thirty-five-day-old-rat castrated at birth. This animal had received 50 mg. of testosterone daily for 5 days. The epithelium is distinctly taller and thrown into folds. The nuclei are stratified ,and there are 5 mitoses in this field. ×365.

adult, and consequently were of no value in determining the age of puberty. In the castrated animals these structures at 10, 20 and 30 days were histologically like the prostate glands of the intact animals of the same ages. At 40 days castration signs, consisting of decrease in epithelial height and secretory granules and a diminution in acinar diameter (3), made their appearance. These changes became progressively more marked as the age of the castrated animals increased.

The seminal vesicles of the untreated animals (fig. 4) were at all ages essentially like those of the intact animals during the first 30 days of life. The implication is, of course, that the pubertal change in these organs seen at 40 days is dependent upon the testes.

In view of the fact that minimal effective doses were being determined, minimal responses in the treated animals were sought. These responses lay in the epithelium of the seminal vesicle and consisted of folding, slight increase in height, stratification of the nuclei, and mitoses. A response of the seminal vesicle to testosterone is shown in fig. 5.

The data are summarized in table 1. The solid line indicates a definite response;

TABLE 1. WEIGHT OF ACCESSORY GLANDS OF REPRODUCTION AND HISTOLOGY OF THE SEMINAL VESICLES IN RATS OF DIFFERENT AGES CASTRATED AT BIRTH AND RECEIVING DIFFERENT AMOUNTS OF TESTOSTERONE

Age, days	Dosage, in micrograms												
	0	5	10	15	20	25	30	35	40	45	50	60	80
	Weight of accessories (mg.) (individual animals)												
10	18 19 8.5		23.5	22	25	20	26 16	23.5	27 29	24.5	27	12.5	[1]
20	29 20 21 27	22	27	20	24	31 21	28 45	31	40 23 42	25.5	40	61	50
30	25 30 25	24	28	26	27 31	29 52.5	38 36 30	40	42		53.5		
40	20 18 21.5 24	12 26 26	26 24	19.5 35	26 28	24.5	24 36		39		38		
50	32 32.5 24	36 53	40 28.5	43 33	42	36.5	33	73				45	
60	18 21 31.5	13	25	19	24	14 37.5	28		+[2]			+[2]	+[2]
80	22.0 21.5		24	20	36	20	27		43				
100	26 17		27		24	25	29		67	31			
120	25.5			24	23.5		28.5	30	31		28		

[1] Solid line denotes a definite response in the seminal vesicle epithelium. Broken line indicates an equivocal response or one in which only part of the group participated.

[2] While weights were not taken, these accessories were obviously larger than those of the untreated animals.

the broken line indicates that the response was equivocal, or that while some animals reacted, others did not. The values shown are weights of the accessories.

On the 15th day the seminal vesicles of all of the animals receiving 35μg. or more from the 10th day showed a definite epithelial response, and in both animals receiving 30μg. there was a detectable response. In none of the animals receiving 25μg. or less was there any histologic response in the seminal vesicles. On the basis of increase in weight of the accessories, 30μg. appears to be the critical dose level. Similarly, in the 20 to 25-day period no animal receiving less than 30μg. revealed histologic response in

the seminal vesicles, while all doses 30μg. or greater elicited histologic reaction and increase in weight. The minimum effective dose in the 10 to 15 and 20 to 25 day periods was thus 30μg.

In the 30 to 35-day period all of the animals, except for one equivocal value at 25 μg., receiving 25μg. or more showed an increase in the weight of the accessories, while less than 25μg. could be regarded as ineffective in this respect. No dose less than 25 μg. had any effect upon the epithelium of the seminal vesicles, while all amounts greater than 35 μg. elicited a response. With 25μg. there was one slight and one good

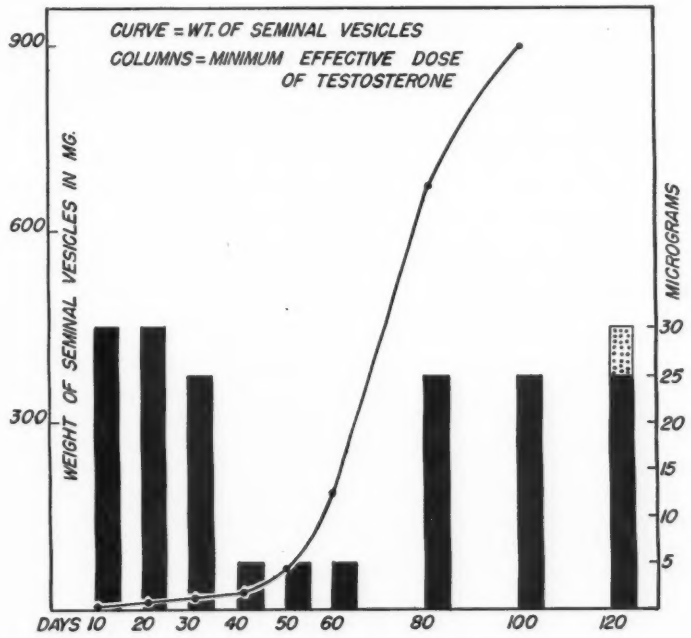


Fig. 6. GROWTH OF THE SEMINAL VESICLES OF INTACT RATS (*curve*), AND THE MINIMUM EFFECTIVE DOSE OF TESTOSTERONE AT DIFFERENT AGES IN RATS CASTRATED AT BIRTH (*columns*). At 120–125 days the threshold is not known; 30μg. produced a response and 20μg. was inadequate. The animal receiving 25μg. was not completely castrated.

epithelial response, and with 30μg. one response was good while two were slight. Thus the threshold during the 30 to 35 day period was 25μg., although the demarcation between effective and ineffective amounts was not as sharp as at 10 and 20 days.

During the 5-day periods beginning at 40, 50 and 60 days all of the treated animals revealed epithelial response in the seminal vesicles, the responses at 50 days being quite marked. On this basis the minimum effective dose at these ages did not exceed 5μg. (the smallest dose tested) and may be less, especially at 50 days. At 40 and 50 days there was weight increase at all of the dose levels, while at 60 days the weights showed no definite increase with doses below 25μg.

In the 80 to 85-day period the accessories were heavier at 20μg. and unaffected at 25μg., while there was distinct epithelial response at 25μg. and a possible effect at 20μg. At 100 days 30μg. was the smallest dose to effect a change in weight of the ac-

cessories, while 25μg. elicited histologic response in the seminal vesicles. At 120 days 30μg. was the smallest dose producing effect upon both weight and histology. Here the animal receiving 25μg. was found to have a testicular fragment, and consequently provided no information as to the effectiveness of this level of androgen.

Thus, as adjudged by epithelial response in the seminal vesicles and to a lesser extent by heavier weights of the accessory glands of reproduction, the minimum effective daily dose of testosterone in male rats castrated at birth was 30μg. during the 5-day periods beginning at 10 and 20 days of age. From 30 to 35 days the daily amount required was 25μg. For the 5-day periods beginning at 40, 50 and 60 days of age not more than 5μg. was required. The data for the same interval beginning at 80, 100 and 120 days are not sufficient to define precisely the minimum required level, although it is clearly not less than 20μg. daily nor more than 30μg., and may be considered to be 25μg. When these values are plotted (fig. 6) it is seen that the period of greatest modification in the histology and the rate of growth of the seminal vesicles in the intact animals was likewise the period of lowest threshold to injected testosterone, that is, of highest reactivity to this androgen.

As would be expected, the response of the epithelium of the seminal vesicles to any universally effective amount of testosterone was enormously greater at 40, 50 and 60 days than at any other age. The prostatic epithelium was not significantly altered by any dose level in the animals not over 30 days of age. In all of the animals 40 days or older, when castration changes became apparent in the untreated animals, the lowest dose evoked a definite response in the prostatic epithelium. A response in this epithelium to 5μg. would be expected on the basis of Hansen's (7) figure of 0.15 capon unit as the smallest daily amount of testicular androgen required in adult rats to elicit a more marked response in the prostate. The coagulating glands were likewise affected by all amounts tested, although the response was not entirely uniform.

DISCUSSION

It is possible that in animals castrated at birth the reactivity at various ages to androgen is not identical with that in intact animals. The only means at present available, however, for determining action of androgen consists in preventing or repairing the changes attendant upon testicular deficiency. In this study the prevention of castration changes was not a possible procedure since there were no recognizable changes upon castration of the animals less than 40 days of age. Similarly, the repair of changes resulting from castrations performed a uniform period preceding the age of testing was impossible since the seminal vesicles would show complete 'castration atrophy' in animals less than 40 days of age, whereas in older animals these structures would be in a state of incomplete involution. Apparently, therefore, animals castrated at birth constitute the most favorable and comparable test objects for the present purpose. There are, of course, no data at hand relative to whether the past action of androgen has any influence on the later action of this hormone in the rat.

It is also possible that the threshold to androgen remains essentially the same throughout life, and that the different thresholds to injected androgen were due to fluctuations in the production of extra-testicular androgen, especially of adrenal origin. The adrenal glands apparently secrete an androgenic substance in early life which is responsible for the secretory state of the prostatic epithelium in infantile, castrated rats (8, 9). The fact that this epithelium undergoes castration changes in castrated rats at precisely the period when the threshold to injected androgen is lowest seems to exclude the possibility of the low threshold representing merely a high titer of extra-testicular androgen.

Differences with age in the capacity of various animals to react to hormones have

been reported from time to time. For example, it is well known that the reaction of immature female rats to gonadotropic substances increases as the age of the animal increases (10, 11, 12). Estrogen will elicit female feathering in the male fowl after the age at which sex differences in plumage are normally established, but is ineffective in this respect in younger males (13). In female guinea pigs spayed at birth the adult threshold of sensitivity to estrogen in inducing estrous behavior is reported by Wilson, and Young (14) to be present at 30 days of age, while twice as much estrogen was required at 20 days, and at earlier ages still larger amounts were ineffective.

On the basis of threshold of response of the seminal vesicles to testosterone, the the rat at 40, 50 and 60 days of age is approximately 500% more reactive to androgen than at 10 and 20 days of age. This difference is more marked when the dose is computed in relation to body weight. It follows, therefore, that the first, and most marked, change from the prepubertal state could occur without increasing the low level of androgen presumed to be present before puberty. Since there appears to be, at least in the bull, no conspicuous increase in level of production of androgen preceding or during this phase (1), the immediate underlying cause of the many patent changes at puberty appears to be a marked increase in responsiveness to androgen. If this finding is of general application, delayed or incomplete attainment of masculine traits may represent, instead of testicular dysfunction, a failure to experience the pubertal increase in responsiveness to androgen. Massive administration of androgen would, of course, exceed a high threshold and produce the effects commonly reported; but such treatment would not be directed at the fundamental disorder. It is interesting to speculate that if the factors responsible for the increased sensitivity were known, the sensitivity might possibly be modified temporarily under appropriate treatment, with consequent permanent correction of the deficient masculinity.

The postpubertal return of the threshold to injected androgen to the prepubertal level rather emphasizes the importance of increased sensitivity in producing the changes characteristic of puberty, especially since less androgen is required in the castrated rat to maintain the normal adult type of seminal vesicle than to repair castration changes once they have appeared (7). Moreover, in the fully grown animal the level of production of androgen has increased greatly over that in the young animal (1), doubtless sufficient to exceed a high threshold. Whether the responsiveness is maintained throughout life at the postpubertal level or changes in old age is as yet unknown. A change in responsiveness may be involved in senility, although decline in androgen production may be the responsible factor. It is interesting that the change in sensitivity to estrogen observed in guinea-pigs by Wilson and Young (14) was permanent rather than limited to the period of puberty. Perhaps acquisition of the ability to exhibit mating behavior under the influence of sex hormones represents a phenomenon fundamentally different from that encountered in the present study.

Whether the observations as to the reactivity of the seminal vesicle to androgen at different ages apply only to this organ or to the body as a whole would probably be difficult to determine. The observations of Price (15) indicate that the behavior of transplanted seminal vesicles in the rat depends on the age of the host rather than upon the age of the grafted tissue or of the donor. If the factors producing the pubertal increase in responsiveness were resident in the seminal vesicle specifically, this increase would presumably be dependent upon the state of development of the seminal vesicle, and would be expected to retain the pubertal level once it is acquired. The observation that the responsiveness later decreases to the prepubertal level suggests that perhaps the mechanism responsible for the pubertal change in reactivity lies outside the seminal vesicle, and may be hormonal in nature.

Whatever the mechanism or factors producing the change in responsiveness may

be, their modification may account for the low level of reactivity to estrogen reported (16) in female rats receiving early treatment with androgen. At present there are two means of inducing precocious development of male sex characters. One is the administration of androgen; the other is the administration of gonadotropic substances. The gonadotropic preparation is considered to stimulate the testes to increased production of androgen, which in turn elicits the somatic responses observed. It may be pertinent to inquire whether increased production of androgen is the only relevant action of the injected material.

SUMMARY

In the colony of rats maintained in this laboratory spermatogenesis becomes complete at approximately 40 days of age. The seminal vesicles at 10, 20 and 30 days exhibit a histologic structure essentially like that of the castrated adult animal. At 40 days the rate of growth of the seminal vesicles increases sharply and their epithelium reveals a beginning of response to androgen, consisting of heightening of the epithelium, appearance of secretion granules, and mitoses. By 60 days the seminal vesicles are histologically fully mature. Puberty may thus be said to begin at approximately 40 days of age.

To determine if there is pubertal alteration in sensitivity to androgen, the minimum effective dose of testosterone was determined at ages 10, 20, 30, 40, 50, 60, 80, 100 and 120 days in male rats castrated at birth. At 10 and 20 days the smallest dose eliciting recognizable response in the seminal vesicles was 30μg. and at 30 days 25 μg., whereas at 40, 50 and 60 days 5μg. was effective. The threshold rose to 25μg. at 80, 100 and 120 days. Thus the rat castrated at birth exhibits greatest responsiveness to injected testosterone at the age at which the accessory organs of reproduction normally undergo their most profound alteration.

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EFFECTS OF ESTROGEN ON ANDROGENIC STIMULATION OF THE PROSTATE AND SEMINAL VESICLE OF THE RAT*

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RESTORATION OF NORMAL structure in the prostate and seminal vesicle of castrate animals is considered to be specifically a function of androgens. This 'restoration of normal structure' includes more than just weight stimulation since androgens stimulate the development of histological and cytological characteristics similar to those of the normal prostate and seminal vesicle (1, 2).

It is also known that the female hormone will cause a weight increase in the sexual accessories of the castrate male. This was first demonstrated by Freud (3) for the seminal vesicles and has been confirmed subsequently by many observers. Freud also claimed that the addition of estrogens to administered androgens caused a marked increase in effect on the seminal vesicles over that obtained with the androgens alone. This greater effect was more than "a simple addition of the separate effects of the two substances." He concluded, therefore, "that estrogens and androgens mutually reinforce each other." Overholser and Nelson (4) obtained similar results and concluded that estrogens and androgens acted synergistically in causing this weight increment. Korenchevsky and Dennison (5) found that the addition of estrogen greatly increased the weight-stimulation effect of androgen on the seminal vesicle, but had only a slight effect, if any, on the prostate. They considered the effects of estrogens and androgens on the sex structures as "co-operative."

In the experiments cited, either the estrogen or the androgen or both were in the form of relatively crude extracts. More recently, however, Korenchevsky and Dennison (6) used crystalline estrogens and androgens on small groups of animals and came to essentially the same conclusion as before. Tschopp (7) also used crystalline hormones and found (in small groups of animals) that estrone added to testosterone caused a greater increase in prostate and seminal vesicle weights than did testosterone alone. Castillo and Pinto (8) found that combined estrone and testosterone propionate caused a greater weight increment in the seminal vesicle than did testosterone propionate alone.

On the other hand, Callow and Deanesly (9) found that administration of estrone and androsterone together had no greater effect on the prostate and only a slightly greater effect on the seminal vesicle than did androsterone alone. This slightly greater development of the seminal vesicle they considered to be due to acceleration of the earliest stages of seminal vesicle growth by the development of connective tissue. Moore and Price have maintained that androsterone (10) testosterone or testosterone propionate (11) administered alone, is capable of maintaining or restoring normal

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structure and function of the seminal vesicle and prostate. They reject the idea that estrogen is necessary as a pacemaker or synergist for male hormone in restoration or maintenance of the castrate accessories.

The present report concerns the effects on the castrate prostate and seminal vesicle of androgens and estrogens, when administered alone and when given together in varying proportions.

PROCEDURE

Seventeen-day-old male rats from 21 litters were castrated. Forty-five days later, litter mates were distributed as uniformly as possible into various groups as follows: one untreated castrate group; one group treated with androgen alone (androgen controls); one treated with estrogen alone (estrogen controls) and four treated with both androgens and estrogens. An additional group was set aside from the original litters. These animals were not castrated and served as normal controls.

The substances used,[1] the daily dosages and the number of animals in each group

TABLE 1. RESPONSE OF SEMINAL VESICLE AND VENTRAL PROSTATE OF RATS TO ESTROGEN AND ANDROGEN

Group no.	No. of animals	Daily test. prop.	Daily α est.	Body Weight		Prostate Weight		Sem. Ves. Wt.	
				Mean	σ	Mean	σ	Mean	σ
		gamma	*gamma*	gm.		gm.		gm.	
1	12	50	—	201.2	9.4	197.8	13.1	439.5	26.3
2	12	50	0.1	189.7	6.3	163.3	8.3	402.2	20.0
3	12	50	1.0	191.3	11.6	182.7	10.3	428.8	30.0
4	12	50	10.0	183.8	8.9	176.6	12.6	430.0	21.9
5	10	50	100.0	160.8[1]	6.0	120.2[1]	8.0	447.2	26.3
6	9	—	100.0	157.8[1]	6.9	7.9	0.97	65.3	3.2
7	8	Castrate	Controls	191.3	11.6	4.5		7.0	
8	11	Normal	Controls	180.2	9.9	207.1	22.9	574.8	56.4

[1] Values significantly smaller than those in *group 1*.
σ=Standard deviation of the mean.

are shown in table 1. Treatment was given daily for 21 days. The single or combined hormones were administered subcutaneously in 0.1 cc. of peanut oil. The animals were killed the day after the last treatment. Seminal vesicles and ventral prostates were removed and immediately weighed. The untreated castrate controls and the normal controls were autopsied at the same age and in an identical manner as the treated animals.

RESULTS

The mean body weights and the mean prostate and seminal vesicle weights for the various groups with their respective standard deviations are presented in table 1.

Body weight. The mean body weights of the various groups were approximately equal at the beginning of the treatment period. At autopsy the mean body weights of the 2 groups given the largest doses of estrogens (*groups 5* and *6*) were significantly smaller than those of the remaining treated and control groups. The body weights of these latter groups did not vary significantly. The low body weights of the 2 former groups were probably due to the 'toxic' effect of high doses of estrogen, an effect which has been noted by many observers. Korenchevsky and Dennison (6) have specifically called attention to the low body weight gain or even loss in groups treated similarly with estrogens.

[1] Testosterone propionate (Perandren) and α estradiol (Ovocylin) were furnished by Ciba Pharmaceutical Products, Inc., Summit, N. J.

Prostate weight. As may be noted in the table, the daily dose of 50γ of testosterone propionate (*group 1*) was approximately a 'physiological dose' in that the prostates were about the same size as those of the normal untreated controls (*group 8*). On the other hand, treatment with 100γ daily of estradiol alone (*group 6*) caused only a slight increase in prostate weight over the castrate control level. This increase was of doubtful significance. The addition of varying amounts of estrogen to the 50γ of testosterone propionate (*groups 2* to *5*) had neither an additive nor a synergistic effect. The mean prostate weight of each of these groups was less than that of the group given the androgen alone. This weight depression was significant, however, only in the group receiving the highest dose of estrogen (*group 5*), in which case the prostates were 39% below the androgen control level.

Seminal vesicle weight. The seminal vesicles of the androgen controls (*group 1*), while somewhat smaller than those of the normal controls (*group 8*), were not significantly different. The 50γ daily dose of testosterone propionate can therefore be considered approximately a physiological dose for the seminal vesicles. The estrogen alone (*group 6*) caused a definite increase in seminal vesicle weight over the castrate level (*group 7*). In none of the groups receiving the combined estrogen and androgen was the seminal vesicle weight significantly greater than with the androgen alone. There was thus no synergistic or additive effect apparent. On the other hand, unlike the effects on the prostate, the added estrogens did not depress the androgen-stimulating effect on the seminal vesicle weight.

COMMENTS

In presenting data on treated animals the resultant organ weights are frequently 'corrected' for body weight by comparing groups on the basis of organ weight to body weight ratios. This procedure may be valid in comparing organs of *untreated* animals or of treated animals which are given a certain dose per gram of body weight, but in both instances, only if a significant correlation between body weight and organ weight is known to exist. When all animals receive the same dosage, however, the smaller animals receive a larger amount of the administered substance per gram of body weight than do the larger animals. Errors introduced by this fact are not corrected, but are exaggerated by presenting and comparing the results of treatment on the basis of organ weight/body weight ratios. This subject is mentioned because the widespread custom of 'correcting' for body weight, even though the administered dose of a substance is constant, may lead some readers to doubt the validity of some of the conclusions presented here.

Specifically, the prostates of the group receiving the highest added dose of estrogen (*group 5*) were significantly smaller than those of the group receiving androgen alone (*group 1*). It might be argued that one cannot conclude that the estrogen caused this prostatic weight depression since the body weights of the animals in this group were also significantly smaller than those of the group receiving androgen alone. This objection, however, is not logical. Due to the 'toxic' effect of the estrogen, the body weight gain of this group was inhibited during the treatment period. As a consequence these animals actually received a larger amount of androgen per gram of body weight and their prostates were therefore exposed to a higher concentration of androgen than were those of the androgen controls. In spite of this apparent advantage their prostates were significantly smaller. It is therefore even more evident that the estrogen inhibited the weight-stimulating effect of the androgen on this gland.

There is a quantitative limitation to the ability of estrogen to inhibit the weight-stimulating effect of androgen on the prostate. The 3 lower dosages of estrogen (*group 2, 3,* and *4*) did not significantly modify the stimulatory effect of the androgen on the

prostate; not until 100γ were given was the effect of the androgen (50γ of testosterone propionate) significantly depressed. The use of insufficient dosage is, according to our observations, the reason Moore and Price (20) found that estrogen did not antagonize the effects of the androgen in the castrate male rat.

There is excellent evidence that estrogens stimulate growth of the fibro-muscular tissue in the prostate and seminal vesicle (3, 4, 12–17) and can thus increase the weights of the prostate and seminal vesicle over the castrate level. In such estrogen-modified accessories, however, there is no stimulation of the secretory function. The epithelium and epithelial structures remain atrophic or undergo metaplastic changes. This latter effect of the estrogens can be antagonized or prevented by adequate doses of androgens (13, 18, 19).

In the presentation of the results, attention has been called to the fact that approximately physiological amounts of androgen were given to the experimental animals. This fact may explain why our results differ from those of other workers who contend that estrogens have an additive or synergistic effect with androgens on the sexual accessories; these latter claims were based on results which, judging by the weights of the sexual accessories, were obtained by using minimal amounts of androgens.

SUMMARY AND CONCLUSIONS

Castrated male rats have been treated with androgen, estrogen and with androgen combined with various amounts of estrogen. The estrogen alone caused a slight weight increase in the prostate and a marked weight increase in the seminal vesicles. The dosage of androgen used, produced prostates and seminal vesicles which did not vary significantly in weight from those of normal animals. The addition of estrogen to this amount of androgen had neither a synergistic nor an additive effect; the seminal vesicles did not vary significantly in weight from those of the group receiving the androgen alone. With the largest dose of esotrogen the prostate weight was significantly depressed below the level caused by androgens alone. These results differed from those obtained by previous workers who used smaller amounts of androgen. The fact that a larger, physiological amount was used in the present procedure may explain the difference in results.

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STUDIES ON RECOVERY OF THE REPRODUCTIVE SYSTEM OF THE MALE RAT FROM REGRESSIVE CHANGES INDUCED BY STILBESTROL[1]

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IN A RECENT PUBLICATION (1) the deleterious effects of stilbestrol on the male reproductive system were discussed. Since considerable regressive changes were found in the testes, seminal vesicles and prostate it was obviously desirable to ascertain not only if these organs could recover a functional level but also to determine the length of time necessary for this recovery after the withdrawal of treatment. With these objectives in mind experiments designed to elucidate the problem were executed. Data collected from these studies are presented in this communication.

MATERIALS AND METHODS

Sixty-six (32 male and 34 female) young but sexually mature albino rats of Wistar stock inbred for many generations were used for the work. Their diet consisted of Purina Dog Chow and water. The sodium salt of stilbestrol was made by dissolving 20 mg. of stilbestrol in 0.5 cc. of 1N NaOH. The desired concentration was attained by dilution with distilled water. One mg. of stilbestrol in 1 cc. of solution was given to 17 male rats 3 times weekly by stomach tube employing the technic described in a previous paper (2). The average period of treatment was 58 days. After several weeks of treatment the rats had the appearance of castrated animals, the scrotum being shrunken and the testicles receded into the abdominal cavity. Upon cessation of treatment one testicle was removed and each rat placed in a separate cage with two female rats. These females were young animals many of whom had raised a litter of young just previous to this test work. Each male was allowed to remain with his consorts until pregnancy was assured or sufficient time had elapsed to show that he was unable to sire a litter. At autopsy the animals were killed by decapitation and the organs dissected free of adherent tissues and immediately weighed. As a means of comparing the organ weights 15 comparable male rats were used as controls and the data were subjected to statistical analysis. A value of 3 or more obtained by dividing the difference of two means by the standard error of the difference was taken to signify that the difference between the two groups was real and not due to chance (table 1). This ratio will be referred to as the significant ratio.' For further study of this problem histological sections were made from the testicles of representative animals, including all rats that failed to sire young, both before and after the recovery period.

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[1] The authors thank Burroughs, Wellcome and Co. New York City for material aid furnished for these experiments.

RESULTS

From table 1 it may be seen that the average weight of one testicle removed immediately upon cessation of treatment was 631 mg. while at autopsy following the intervening recovery period the average weight of the remaining testicle had risen to 1,368 mg. An analysis of these data further revealed the notable recovery of the testicle as shown by a significant ratio of 6.7 (not shown in table 1). The results of the comparison of the above values with untreated control rats are shown in table 1. A significant ratio of 10.8 indicates that the testicles of the treated rats were substantially reduced in weight by the treatment while after recovery the weight of the testicle was not significantly different from that of the untreated control animals, the significant ratio being only 0.42 (table 1). This latter finding was also true

TABLE 1. MEAN ORGAN WEIGHT OF 15 MALE RATS TREATED WITH STILBESTROL AND 15 CONTROL RATS
Organ wt., mg.; body wt., gm.

	Mean Weight		Mean Diff.
	Control	Treated	S.E. of Diff.
Testicle	1402	631[1] 1368	10.8[1] 0.42
Epididymis	502	476	0.94
Prostate	616	571	0.76
Seminal vesicles	999	1022	0.24
Body wt. at autopsy	330	342	—

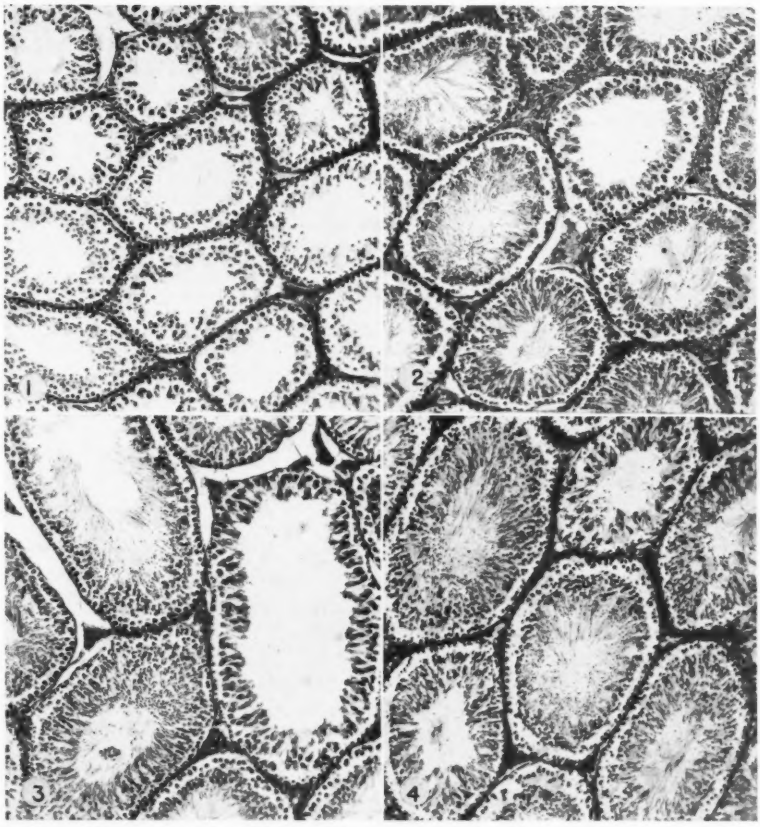
[1] Top figure represents value before recovery, bottom figure after recovery.
S.E. of Diff. $=\sqrt{(SE_1)^2+(SE_2)^2}$
S.E. = S.E. of Mean $=\sigma/\sqrt{n}$

for the remaining reproductive organs investigated. The significant ratios for the epididymis, prostate and seminal vesicles being 0.94, 0.76 and 0.24 respectively (table 1).

To determine the accuracy with which the prostate gland was dissected the relationship between the weights of prostate and the seminal vesicles was calculated. In the case of the control group it was found that the prostate was 38.1% of the combined weights of the seminal vesicles and the prostate gland whereas in the treated group after recovery this was found to be of a similar value, 35.8%, thus indicating that we were consistent in separating these two glands in their proper relationship one to the other.

Of the 17 treated male rats 13 recovered a functional state. The average time interval from cessation of treatment to birth of the young was 68.5 days. Assuming the gestation period of the rat was the normal 21 days it required an average of 47.5 days for the testicle to regain its functional state. Twenty-four of the 34 test females produced a total of 197 young; 96 males and 101 females, with an average of 8.2 young per litter.

The histological study of the testicles sectioned immediately upon cessation of treatment revealed marked degeneration of the tubules comparable with that reported previously (1) (fig. 1). After the recovery period the remaining testicle from those animals which had sired young showed incomplete recovery (fig. 2); however, spermatozoa were present in the majority of the tubules. The testicles from the 4 males which failed to sire young showed less interstitial tissue and more tubular degeneration than the functionally recovered testicle. In 2 of these 4 cases spermatozoa were noted in some of the tubules; however, these may have been immature or ab-



PHOTOMICROGRAPHS OF TESTICLES OF RATS TREATED WITH STILBESTROL. ×67. Fig. 1. Testicle from rat given 3 mg. of stilbestrol weekly for 8 weeks; immediately after withdrawal of treatment. Fig. 2. Testicle from same rat as A, after recovery period of 69 days. Fig. 3. Testicle from rat which failed to sire young; 4 months after cessation of treatment. Fig. 4. Normal testicle from untreated rat.

normal in character (fig. 3). The testicles from the remaining 2 animals contained no spermatozoa whatever.

Since the data pertaining to the organ weights after recovery were taken from semi-castrated animals it is important to note that no hypertrophy was detected when the organs were compared with those of the normal. Nor would hypertrophy of the remaining testis be expected in these nearly mature rats. Lipschütz (3) reported that he found no hypertrophy in evidence when adult rabbits were semi-castrated. The effect of stilbestrol on the epididymides, prostate and seminal vesicles was also found to be temporary. Statistical analysis revealed that weights of these organs after recovery were not different from those of the untreated control group.

DISCUSSION

Despite the fact that many data have been published showing the deleterious effects of various substances and treatments on both the ovary and testicle there

seems to be a paucity of suitable information pertinent to the recovery of these organs from such influences. Moore (4) reported that the degeneration of the testicle produced by high environmental temperature (in abdominal cavity 24 days) was not permanent in nature. He pointed out, however, that all of the tubules were not fully recovered after a 74-day recovery period. Some had epithelium approximately normal in character but no spermatozoa; a few tubules showed no signs of recovery whatever. Emery (5) demonstrated that female guinea pigs did not become pregnant until 4 to 8 weeks after injections of testicular material were stopped. In the case of female rabbits the sterility produced by injections of spermatozoa or testicular extracts was found by Pommerenke (6) to have a duration of 6 to 25 weeks. Zuckerman (7) reported that a rhesus monkey showed, on the 8th day after cessation of treatment, signs of recovery from ovarian atrophy caused by prolonged administration of testosterone propionate. Working with rats Mazer and Mazer (8) showed that the effects of prolonged testosterone propionate treatment on the ovary were transitory in nature. A 70% increase in weight of the ovaries together with follicular growth, ovulation and luteinization was found in the case of animals autopsied 29 days after cessation of treatment. In his studies on the effects of estrogens on male fowl Emmens (9) found that one cock, which was allowed 80 days to recover from a series of estradiol benzoate injections, showed rapid recovery of comb size although testicular weight had not attained a normal range. Histologically the testicles were very nearly normal in appearance.

Stilbestrol may affect the testicle by at least two mechanisms; the first possibility is that of a direct action of the substance on the organ producing an impedance or cessation of the normal physiological processes thus causing abnormal sperm formation or aspermatogenesis. The effect of stilbestrol on the pituitary gland offers a second possibility whereby the testicle may be affected. Since the location of the testicle, either in the scrotum or abdominal cavity, is thought to be under the control of the pituitary, any treatment which would affect the gonadotropic function of this gland might cause atrophy and recession of the testicles into the abdominal cavity and further sterility as a result of the higher environmental temperature.

SUMMARY

Seventeen male rats were treated with 1 mg. of stilbestrol 3 times weekly for 58 days. They were then semi-castrated and each placed with 2 test females to determine the interval necessary to attain a functional level. At the end of treatment all animals were sterile. Thirteen of the 17 male rats recovered sufficiently to effect successful impregnation. The mean time required for the remaining testicle to recover a functional level was 47.5 days.

A statistical comparison of the weights of the reproductive organs after recovery with those of untreated controls revealed no significant weight difference.

Histological studies revealed that only partial recovery of the remaining testicle had occurred in some cases.

The male organs of reproduction have the capacity for gaining rapidly in weight and regenerating new tissue cells following regressive changes induced by stilbestrol.

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EFFECTS OF TESTOSTERONE PROPIONATE IN IMMATURE AND ADULT FEMALE RATS[1]

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A PREVIOUS REPORT FROM THIS LABORATORY dealt with the action of testosterone propionate on the ovaries and uteri of immature rats in five-day experiments (1). The present communication is concerned with the changes occurring in the ovaries, vaginae, uteri and mammary glands of young and adult rats receiving injections of testosterone propionate over long periods of time.

Since Ihrke and D'Amour (2) pointed out that the administration of an extract of testicular tissue resulted in a suppression of the estrous cycles of rats, many reports dealing with this problem have appeared. It has been shown that testosterone propionate is the most active of the androgenic substances in regard to its effects in female animals, but there are many discrepancies in the results reported by different investigators.

There is, however, general agreement that in immature female rats the administration of testosterone induces a premature establishment of the vaginal introitus (1, 3–11). The accompanying state of the vaginal epithelium has been represented in different ways, some workers claiming that estrous changes with complete cornification are the usual finding and others that there is merely a 'mucification' of the mucosa. It is probable that these divergent views can be explained by the fact that with the opening of the vagina there is a short period during which smears show cornified cells (estrus) and subsequently there is a prolonged phase when the smears correspond to the period of diestrus and histologic examination reveals a state of mucification (9–12). Of more importance is the fact that vaginal opening is induced by injections of testosterone after ovariectomy (6, 7) and after hypophysectomy (11). On the other hand, in the normal adult female testosterone causes a suppression of the estrous cycles and the vaginae present the histologic picture of mucification (10, 11, 13–18). Following hypophysectomy there may be one or more periods of cornification (11, 23), but mucification is the rule following this operation as well as after ovariectomy (11, 12, 16).

With 4 exceptions (10, 19, 20, 21) among the reports which have come to our attention, the uteri of both immature and adult rats receiving testosterone have been described as much heavier than those of controls, and characterized by a histologic picture reminiscent of that found during the second half of normal gestation when there is a thickening of the endometrium which is heaped up into characteristic lace-like folds (4, 5, 8, 9, 11, 15, 17, 19, 22). This change has been reported as occurring both in hypophysectomized rats (11, 23) and, with one exception (22), also after ovariectomy (6, 12, 16). Although this uterine condition is held as closely resembling a normal progestational proliferation, it is considered by some as not strictly identical because it does not favor the development of deciduomata following the traumatization of one or the other uterine horn (22, 23, 24).

The most contradictory reports are seen in those dealing with the effects of testosterone on the ovary. According to some authors this hormone in immature rats stimulates the

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growth of normal graafian follicles and the production of corpora lutea (4, 6, 8, 9), while others state that luteinization is absent or only found in exceptional instances and there is merely a limited development of follicles (1, 7, 10, 11). One group of workers reported that the prolonged administration of testosterone begun shortly after birth results in the appearance of gigantic ovaries containing lutein cysts (5).

A similar discrepancy is apparent in studies of ovarian changes in adult rats. The observation most frequently made is that fresh corpora lutea are found and that they show an unusual degree of hypertrophy, thereby suggesting an active endocrine function (13, 15, 17, 19). In one instance the authors state that with testosterone treatment a single set of large corpora appears and with a prolongation of the period of injections follicle cysts are formed (25). One group of investigators reports an atrophy of the ovaries with an absence of corpora after a prolonged period of treatment (10, 21), another that ovulation is inhibited and the

TABLE 1. ORGAN WEIGHTS OF RATS INJECTED WITH TESTOSTERONE PROPIONATE
Age 21 to 23 days at start of experiment

Days injected	Total dose testosterone	No. of rats	Average body wt.	Uterus	Ovaries	Hypophysis
	mg.		*gm.*	*mg.*	*mg.*	*mg.*
30	13	3	82	409	9	3
controls	—	3	105	115	44	5
60	26	3	114	321	7	4
controls	—	3	128	136	38	6
90	39	4	136	338	11	4.5
controls	—	3	151	295	48	8

rats become sterile (18), while one claim is made that follicles may be noted at all stages of development but there is no luteinization and stromal and thecal tissues show the deficiency 'wheel cells' described by Selye as occurring after hypophysectomy (26). In both immature and adult hypophysectomized rats testosterone was without effect on the ovary (11, 27) except possibly in one experiment where it was reported that the ovaries were smaller but contained corpora lutea (23).

In both normal and ovariectomized rats testosterone causes a hypertrophy and secretory activity of the mammary glands (11, 15, 17, 27), but this change does not occur after hypophysectomy (11, 28). One group emphasizes an increase of fibrous tissue succeeded by the formation of cysts (29).

METHODS

The animals employed in this study were the offspring of the inbred Stanford colony of white rats which has been maintained for many years. The immature females were 21 to 23 days of age and the adults a few days more or less than 90 days old at the beginning of each experiment.

The testosterone propionate[2] was made up in sesame oil containing 10 mg. per cc. The injections were given subcutaneously. In the case of the immature animals 1.0 mg. was administered 3 times a week. Twenty-two rats were employed. Three received 13 mg. of testosterone propionate over a period of 30 days, 3 were given 26 mg. for 60 days, 3 were given 39 mg. for 90 days, and 3 were treated with a preparation of chorionic gonadotropic hormone after the injection of 39 mg. over 90 days. This gonadotropin (*P.B.E. No.* 94) was a crude but potent extract of blood of pregnant women prepared in the manner described in a number of previous com-

[2] Testosterone propionate (Perandren) supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

munications (30, 31). The animals were given 0.25 cc. of the extract subcutaneously twice daily for 4 days and killed on the 5th day. In immature rats the same extract given in the same manner produced ovaries with large corpora lutea and follicles and their weight was somewhat more than twice the normal weight. Ten littermates receiving injections of plain sesame oil were reserved as controls (table 1).

The adult females uniformly received 4 mg. on the 1st day of each experiment and then every second day until a total of 40 mg. had been administered. They were killed on the 20th day, that is, 24 hours after the last injection. The control figures were

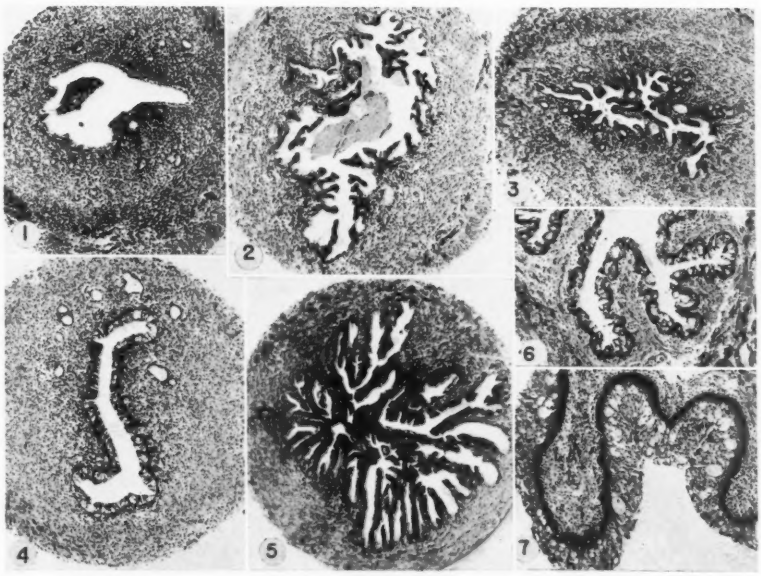
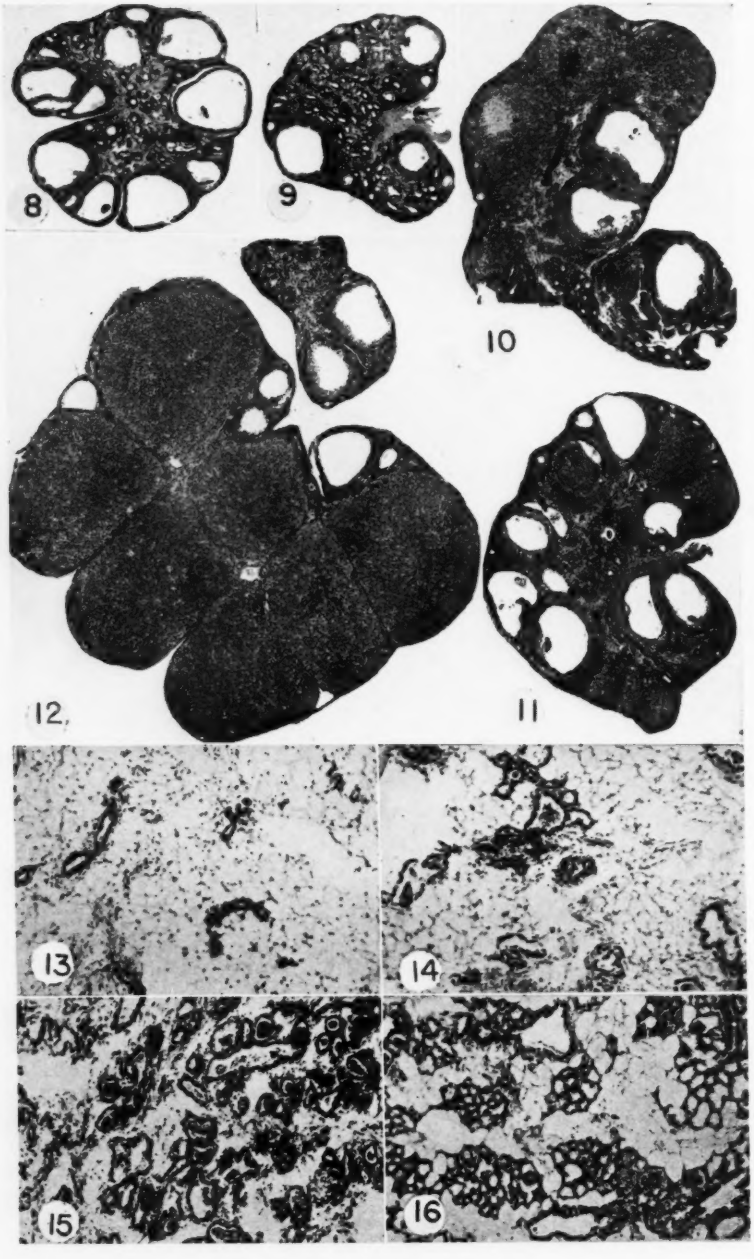


Fig. 1. Uterus of normal adult rat. Fig. 2. Uterus of rat, age 51 to 53 days, treated for 30 days with testosterone. Fig. 3. Uterus of adult rat which received first injection of testosterone during diestrus. Fig. 4. Uterus of adult rat which received first injection during estrus. Fig. 5. Uterus of rat age 111 to 113 days, treated with chorionic gonadotropin following injections of testosterone over a period of 90 days. Fig. 6. Mucification of vagina of rat receiving first injection during diestrus. Fig. 7. Vaginal mucosa of adult rat receiving first injection at estrus.

obtained from uninjected animals of nearly the same age, half of which were killed during diestrus and the remainder during estrus.

Three series of adult female rats were treated with testosterone propionate in the manner described above. The first 2 groups consisted of 11 and 5 rats respectively, but the results are not given in detail because the stage of the estrous cycle at which the injections were begun was not known. This report is based on a 3rd group composed of 10 rats which had been carefully studied with daily vaginal smears for 10 days before the injections were begun. In *group A*, 5 animals received the first injection when the vaginal smear corresponded to the diestrus phase, that is, it was characterized by the presence of nucleated epithelial cells and leukocytes. In *group B*, the rats were given the first injection of testosterone at estrus, when the vaginal smear showed cornified cells and there was a complete absence of leukocytes.



Figs. 8–16. See opposite page for legend.

RESULTS

The results of our experiments may be considered under 3 headings according to the type of animal employed, namely, *a*) immature rats, *b*) adult females in which the injections were begun at diestrus, and *c*) adult females in which the first injection was administered during estrus.

a) *Immature rats.* In a previous communication (1) the effects of testosterone propionate administered to young female rats over a period of 5 days were reported, and they were in accordance with the work of several other investigators. The injection of as much as 45 mg. failed to produce any notable increase in ovarian weight, but follicle growth was demonstrable. Histologically, the uteri were markedly increased in size, and the vaginae showed extensive mucification.

The results obtained in the immature rats injected for periods of 30, 60 and 90 days essentially conform to those found in the group treated for only 5 days. It is seen from table 1 that the testosterone prevented the development of the gonads, since the ovaries of the treated rats always weighed less than those of the controls which received plain sesame oil. In fact, after the injection of 39 mg. in 90 days, when the animals had reached the age of 111 to 113 days, the ovaries only weighed 11 mg. (controls, 48 mg.), a figure which corresponds to the average found in our colony in 21-day old immature females. On the other hand, the uteri were consistently enlarged and the body weight also was affected so that the experimental animals weighed less than those receiving only plain sesame oil. The hypophyses in each group of this series weighed slightly less than those of the controls.[4]

The histologic appearance of the vaginae, ovaries, mammary glands, and uteri was consistent at all stages of the experiment. After 30 days the ovaries were characterized by a marked increase of fibrous tissue in the stroma, and a gonadotropic effect was seen in the presence of follicles at different stages of development or undergoing atresia (fig. 8). In all the sections studied only one corpus luteum was seen. The ovaries of the animals treated for 60 and 90 days presented the same changes but the fibrosis was much more pronounced and there were a few 'deficiency cells,' described by Selye as occurring after hypophysectomy. Follicle growth and various stages of atresia were still evident and there were no corpora lutea (fig. 9).

The uteri of all the animals were hypertrophied and the endometrium gave a picture of hyperplasia in which the surface epithelium assumed a peculiar branching conformation (fig. 2). The subepithelial tissues were moderately infiltrated with leukocytes, but there were no eosinophiles.

The vaginal introitus was established early in the experiment and at all stages showed extensive mucification. The histologic examination of the mammary glands was of interest since there was a definite proliferation of the ducts, secretion, marked fibrosis of the intertubular tissues, but only slight alveolar stimulation (fig. 14).

[4] The anterior pituitary glands of representative numbers of the immature and adult rats used in these experiments have been studied as to their cytological behavior. The results will be presented in detail in a future communication.

Fig. 8. Ovary of rat 51 to 53 days old, given testosterone for 30 days. Fig. 9. Ovary of rat, 111 to 113 days old, given testosterone for 90 days. Fig. 10. Ovary of rat given chorionic gonadotropin following 90 days of treatment with testosterone. Fig. 11. Ovary of adult rat; first injection during diestrus. Fig. 12. Ovary of adult rat; first injection during estrus. Fig. 13. Mammary gland of normal adult control. Fig. 14. Mammary gland of rat 51 to 53 days old, treated with testosterone for 30 days. Fig. 15. Mammary gland of adult rat which received first injection of testosterone during diestrus. Fig. 16. Mammary gland of adult rat; first injection during estrus.

Chorionic hormone after testosterone injections. Three of the rats which had received 39 mg. of testosterone over a period of 90 days were given 8 injections of chorionic hormone and were killed 120 hours later as described in the preceding section of this report. At autopsy there was a marked increase in the weights of the ovaries (35 mg.) and the uteri (402 mg.), as compared to the testosterone-treated group and the normal controls (table 1). The ovaries histologically presented the picture usually found following the administration of chorionic hormone to immature rats. The stroma had completely lost its shrunken fibrotic appearance and was made up of hypertrophied cells (fig. 10). There were no atretic follicles but numerous developing graafian follicles, many corpora lutea, and lutein cysts with and without intracystic hemorrhages (Blutpunkte). The vaginae were in a state of mucification. The most startling finding was in the endometrium, which presented an

TABLE 2. ORGAN WEIGHTS OF ADULT RATS INJECTED WITH TESTOSTERONE PROPIONATE. Experiment begun during diestrus in one group, and during estrus in another

Total dose testosterone	Time first injection	Time of autopsy	No. of rats	Average body wt.	Uterus	Ovaries
mg.				gm.	mg.	mg.
40	diestrus	—	5	163	460	26
controls	—	diestrus	5	163	281	60
40	estrus	—	5	169	586	69
controls	—	estrus	5	182	396	76

appearance strikingly similar to the progestational endometrium of the rabbit (fig. 5). The lining epithelium was composed of low cuboidal cells and the mucosa was not only thrown up into numberless folds but assumed a tree-like branching conformation. The subepithelial tissues were dense and there was no leukocytic infiltration. We do not know of any previous report of a similar histologic transformation of the rat's endometrium.

b). Adult rats; diestrus. The 2nd group consisted of 5 adult rats treated for 20 days in which the first injection was given during diestrus. The results closely parallel those found in the immature animals.

The ovaries were small, averaging 26 mg. in weight as compared to controls of 60 mg. (table 2). On histologic examination there were old but no definitely recent corpora lutea, and a number of follicles at various stages of development or presenting different degrees of atresia (fig. 11). The uteri weighed more than those of the controls, and histologically were directly comparable to those of the immature series in that the endometrium was hyperplastic and thrown up into branching folds as previously described (fig. 3). The vaginal epithelium showed varying degrees of mucification (fig. 6).[5] The mammary glands in this group also corresponded to those of the immature animals except that the characteristic changes were more accentuated. The nipples were not unduly prominent, while grossly normal amounts of breast tissue were visible and the consistency was of a granular nature. The tubular epithelium showed proliferation and some secretory changes, but only an occasional alveolus had developed and there was a striking increase of fibrous tissue (fig. 15).

c). Adult rats; estrus. The findings in the rats which received the first injection of testosterone during estrus were totally different from those of the other 2 groups.

[5] In both groups of adult animals (first injections at diestrus and estrus) the vaginal smears contained abundant leukocytes and a few epithelial cells for 3 days after the initial injections, regardless of the phase of the cycle at which the experiment was begun. Subsequently, the smears were composed of fewer leukocytes and relatively more epithelial cells with prominent nuclei.

In the first place, the ovaries were large, the average weight being 69 mg. as compared to 76 mg. for the untreated controls and 26 mg. for the diestrous series (table 2). This increase is apparently accounted for by the presence of many unusually large corpora lutea with hypertrophied lutein cells. These structures were striking and made up the main substance of the ovaries, although normal graafian follicles were also evident (fig. 12). The uteri were large, and on microscopic examination showed a hyperplasia of the endometrium but it did not have the branching arrangement noted in the other groups (fig. 4).

The nipples were prominent and the mammary glands were enlarged to such an extent that the change was discernible grossly, as they extended to the midline and far laterally. Histologically there was not only a proliferation of the ducts but definite alveolar formation (fig. 16). The vaginal mucosa was in an accentuated state of mucification, the cells forming many layers with marked vacuolization (fig. 7).

DISCUSSION

In order to explain the discrepancies in the reports of different investigators it has been suggested that they are due mainly to variations in the dosages of testosterone employed and in the duration of the experiments. Although these 2 factors are significant, we feel that a most important consideration is the functional state of the ovaries at the time of the first injection, as originally indicated by Wolfe and Hamilton (13).

Our results fall naturally into 2 groups. In the 1st were the immature rats and the adults with a diestrous vaginal smear at the beginning of treatment. Before treatment the ovaries did not contain mature follicles, the corpora lutea of the adults were undergoing degeneration, and the injection of testosterone produced fundamentally identical results. The vaginae were in a state of mucification; the uteri were enlarged and the endometria hyperplastic and thrown into folds; the ovaries were atrophic except for graafian follicles developing almost to maturity and then undergoing atresia; and the mammary glands showed a growth of the ducts, secretion, and a marked increase of fibrous tissue.

In the 2nd group were the adult rats first injected at estrus, that is, at a time when ovulation normally occurs and active functioning corpora lutea develop in the ovaries. These animals showed changes differing from those of the 1st group. The vaginae were in a state of accentuated mucification and vacuolization; the uteri were large but the histologic picture was not the same; the ovaries were comparatively heavy due to the presence of large corpora lutea with hypertrophied lutein cells; and the mammary glands showed marked proliferation of the acini as well as the tubules.

Any explanation for the mode of action of testosterone in female rats must take into consideration the fact that its effects are produced by direct as well as indirect stimulation. It has been demonstrated already by several competent observers that testosterone acts directly on vagina and uterus, since these changes may be induced in both ovariectomized and hypophysectomized animals.

On the other hand, it also has been recognized that the changes in the ovaries and breasts are dependent on the presence of an intact hypophysis in the experimental animal. The fact that testosterone does act on the anterior lobe is generally conceded, but we are not in accord with the theory that it produces an increased activity of the luteinizing hormone. On the basis of our experiments we feel that the reverse is true, namely, that testosterone inhibits the production of the luteinizing or interstitial-cell-stimulating substance. Except for a single yellow body in one of the immature rats we have found no evidence of corpus luteum formation which could be ascribed to the injections of the male hormone, although the ovaries contained many

large follicles which might have become luteinized. The belief that they retained the potency to undergo luteinization is amply supported by their rapid response to injections of chorionic gonadotropin. Another observation which upholds this contention is the marked atrophy of the interstitium which occurred during the period of injection and which also can be attributed to a lack of the luteinizing factor.

In contrast to the lack of luteinizing hormone, the persistence of a follicle-stimulating influence was seen in the continuous development of graafian follicles, especially noticeable in the immature animals. This picture of growing follicles in small ovaries with a fibrous, atrophic stroma is reminiscent of that observed in the gonads of hypophysectomized rats following the administration of a follicle-stimulating extract prepared from the urine of postmenopausal women (32). The work of Hamilton and Wolfe (33) demonstrating a decrease of the gonadotropic hormone (FSH or LH ?) content of the hypophysis following testosterone injections possibly does not support our theory. However, the fact that the preliminary administration of this substance to immature rats augments the increase of ovarian weight induced by chorionic gonadotropin favors the concept that testosterone stimulates an increased production of follicle-stimulating hormone (1).

Another modification of anterior pituitary function which has been suggested as resulting from the administration of testosterone is an increased production of prolactin. This observation is supported by the fact that testosterone induces mammary changes only in animals with intact hypophyses. Reece and Mixner (34) also have demonstrated that the daily injection of testosterone for 15 days produces a 40% augmentation of the lactogen content of the pituitary glands of adult spayed rats. The increased lactogenic hormone production is also a valid explanation for the maintenance of the large corpora lutea seen in the ovaries of rats first injected at estrus. In recent studies (35, 36) it has been shown that prolactin has a direct action on the ovary, stimulating the corpora lutea to hypertrophy and secrete increased amounts of progesterone.

As stated previously, the uterine changes are brought about by a direct action of testosterone. The hyperplastic endometrium is usually considered as a progestin-like effect and compared to the second progestational phase of Selye, Collip and Thomson (37), but a somewhat similar transformation has been described by Zondek (38) following the administration of large dosages of estrogen. In the case of our rats receiving the first injection of testosterone at estrus the resulting uterine change must be attributed not only to the direct action of the hormone but also to the added influence of the hypertrophied corpora lutea present in the ovaries.

SUMMARY

Three groups of female rats were given subcutaneous injections of testosterone propionate for periods varying from 20 to 90 days.

In immature rats and adults receiving the first injection during diestrus the vaginae had the microscopic appearance of mucification; the uteri were enlarged with the endometrial epithelium heaped up into lace-like folds; the ovaries were atrophic with a fibrous stroma; they contained no recent corpora lutea but many follicles at various stages of development or undergoing atresia; and the mammary glands presented a marked increase of intertubular fibrous tissue with an overgrowth of the ducts and some evidence of secretion.

When the injections were begun during estrus in adult animals the vaginae showed marked mucification and vacuolization; the uteri were large but histologically did not correspond to those of the other 2 groups; the ovaries were large and contained hypertrophied corpora lutea; and the breasts were characterized by enlargement and the proliferation of acini as well as ducts.

The administration of chorionic gonadotropin to young female rats previously treated with testosterone propionate over a period of 90 days resulted within 5 days in an extensive luteinization of follicles and hypertrophy of stromal cells.

The theory is advanced that testosterone propionate in female rats induces a functional transformation of the anterior hypophysis characterized by an inhibition of the so-called luteinizing or interstitial-cell-stimulating hormone and an increased production of prolactin and the follicle-stimulating hormone.

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EFFECT OF SEX HORMONES, SEPARATELY AND COMBINED, ON THE PROLIFERATION AND HYDRATION OF COMBS AND CLOACAE OF MALE CHICKS

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A RECENT EXPERIMENT (1) carried out in this laboratory showed that the sex eminence of the baby chick is at least partly under hormone control. Injections of testosterone propionate into young chicks of both sexes resulted in a considerable enlargement of the organ. This was always accompanied by a 'puffing' reaction which affected the cloacal region (vent). Such cloacae appeared swollen and edematous.

Sex hormones are known to impart to certain tissues water retentive qualities (2–8). It was pertinent to the earlier study, therefore, to determine whether the puffing of the cloacal region involved alterations in water metabolism of the affected tissues. The present paper reports the result of an experiment designed to study the problem from several angles.

MATERIAL AND METHODS

Male baby chicks of the Barred Plymouth Rock and the White Leghorn breeds were used. As soon as they hatched, the chicks were separated accorcing to sex, and the males transferred to an electric battery brooder where they were kept for the duration of the test. An interval of 48 hours was allowed to elapse between the hatching date and the beginning of the experiment.

Each breed was divided into 5 groups according to the treatment which consisted of one of the following. *Group* 1, a total dose of 2 mg. of testosterone propionate per chick;[1] *group* 2, 0.2 mg. of testosterone propionate; *group* 3, 2 mg. of estradiol dipropionate; *group* 4, combination of testosterone propionate and estradiol dipropionate (2 mg. of each); *group* 5, controls, receiving the solvent only. Each chick received 3 injections spaced at about equal intervals during the 10-day injection period. Sesame oil was the solvent, the total dosage of oil being 0.6 cc. per chick. Ten days after the first injection, when the chicks were 12 days old they were weighed, gassed and immediately dissected.

During the period of injection, observations were made on the growth rate of the sex eminence, the papilla of each chick being graded according to an arbitrary scale similar to, but more extended than that used in the previous study (1). These observations were made 48 hours after each of the 3 injections.

In addition to these systematic observations, at the conclusion of the treatment period linear measurements were made of the width of the cloacal opening.

Both the cloacal region and the comb were cut out in each bird and weighed to

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[1] Testosterone propionate and α-estradiol dipropionate were kindly supplied by Ciba Company, Limited of Montreal.

the nearest milligram. To obtain data on the moisture content of these parts, about 10 pairs from each breed group were desiccated to a constant weight at 110°C.

RESULTS

Cloaca and sex eminence. As early as 48 hours after the first injection, the estrogen-treated groups showed signs of puffing in the cloacal region, a phenomenon first described by Wolff (9). There was a corresponding reaction in the androgen-injected birds although on a much smaller scale. The cloacal region continued to respond progressively to the estrogen throughout the duration of the experiment. The whole area presented a picture of extreme turgescence. Both the dorsal and the ventral lips

TABLE 1. EFFECT OF VARIOUS TREATMENTS ON SIZE OF SEX EMINENCE AND ON WEIGHT AND WATER CONTENT OF CLOACA AND COMB IN CHICKS

Group 1, 2 mg. of testosterone propionate; *group* 2, 0.2 mg. of testosterone propionate; *group* 3, 2 mg. of estradiol dipropionate; *group* 4, 2 mg. of testosterone propionate plus 2 mg. of estradiol dipropionate.

Group	breed	Wt. at autopsy	Sex eminence 48 hr. after first inj.	Sex eminence 48 hr. after second inj.	Sex eminence 48 hr. after third inj.	Width of cloaca	No. of chicks[1]	Cloaca Wet wt.	Cloaca Moisture[2]	Comb Wet wt.	Comb Moisture[2]
		gm.	mm.			mm.		mg.	%	mg.	%
1	W. Leg.	83.0±3.1	2.8±0.1	3.3±0.1	4.3±0.1	6.6±0.2	16	199±12		338±29	
							10	179±10	79.2±0.8	313±44	85.9±0.4
	B. Rock	77.8±2.6	2.5±0.1	3.0±0.1	3.6±0.1	6.1±0.2	19	195±11		64± 5	
							10	165±13	78.3±0.8	67± 7	81.1±0.6
2	W. Leg.	78.3±3.3	1.8±0.1	2.6±0.1	2.8±0.2	6.2±0.2	16	169±12		115±12	
							8	176±14	76.4±0.8	136±14	79.6±1.8
	B. Rock	77.2±5.0	2.0±0.2	2.6±0.1	2.8±0.1	5.4±0.2	16	133± 7		25± 3	
							8	127±11	77.2±0.3	23± 3	70.8±1.7
3	W. Leg.	75.8±2.7	2.9±0.2	4.6±0.3	5.4±0.3	11.1±0.4	14	638±37		40± 4	
							8	654±55	83.0±0.4	40± 5	76.9±0.6
	B. Rock	80.8±3.7	3.0±0.3	5.0±0.2	5.7±0.2	10.5±0.3	18	598±43		16± 1	
							10	664±36	82.7±0.6	15± 2	72.9±1.5
4	W. Leg.	83.6±2.8	3.3±0.3	5.5±0.3	6.0±0.2	12.1±0.5	10	590±43	83.0±0.5	385±45	87.1±0.5
	B. Rock	78.3±3.6	3.4±0.2	5.3±0.2	5.9±0.2	10.9±0.3	18	551±66		64± 6	
							10	480±53	83.2±0.5	52± 6	81.3±0.5
5	W. Leg.	83.5±3.2	1.4±0.1	1.9±0.2	2.4±0.2	5.3±0.2	13	126±17		40± 4	
							10	102±11	77.7±0.4	36± 4	74.8±0.7
	B. Rock	89.2±3.4	1.5±0.1	1.8±0.1	2.1±0.1	4.5±0.3	18	121± 9		21± 2	
							10	123± 7	77.9±0.3	18± 3	71.7±0.7

[1] Upper figure includes all chicks; lower figure those on which moisture determinations were made.
[2] Calculated from wet weight.

of the cloaca appeared edematous, with the width of the orifice being considerably greater than in either the androgen-treated or the control chicks.

There was also a qualitative difference in the reaction to the hormones in the 2 groups. Cloacae of the androgen-injected birds, with the swelling largely limited to the immediate lips of the orifice, showed evidence of hyperemia, a symptom almost completely absent in the estrogen groups except in the initial stages of the reaction.

Paralleling the cloacal reaction of the estrogen-treated chicks there was also a tremendous enlargement of the sex eminence. This increase was much greater than that occurring in the groups receiving androgen (table 1). First signs of this differential rate of stimulation by the two hormones appeared on the 4th day of treatment. These initial size differences increased progressively and on the 10th day, when the chicks were killed, the estrogen-treated birds of both breeds had eminences considerably larger than those in the androgen-treated groups. These differences were statistically significant. At the same time, the combination of the 2 hormones failed to produce any

substantial evidence for a synergistic action on the development of the eminence since it can be seen from table 1 that the size of the eminence in *group 4* (2 mg. of testosterone propionate + 2 mg. of estradiol dipropionate) is not appreciably greater than that in *group 3* (2 mg. of estradiol dipropionate).

Comb. The response of the comb tissue in both breeds was the exact opposite to that of the cloacal region. Here testosterone propionate produced a tremendous proliferation while estradiol dipropionate was practically without effect. As would be expected, the groups injected with both hormones showed proliferation of both the cloacal region and the comb tissue. Here again there was no evidence of either synergism or antagonism.

Tissue hydration. The data in table 1 show clearly that the apparent hypertrophy of these 2 hormone-sensitive areas, cloaca and comb, was due in a large degree to the local accumulation of water in the tissue. In both cases any substantial increase in the

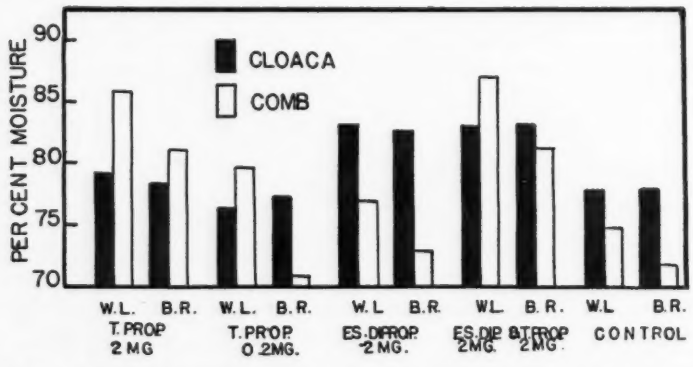

Fig. 1. Percentage of water in the cloacae and combs of control and injected groups of chicks. Showing the hydration of the combs due to testosterone propionate and of the cloacae due to estradiol dipropionate and of both under combined hormone injections.

wet weight of the tissue was accompanied by an increase in both the absolute and relative amounts of moisture. The relative percentages of moisture in the cloaca and comb in the various experimental and control groups are shown in figure 1.

As for the cloaca, only in the groups receiving estrogen and estrogen plus androgen was there clearcut evidence of water retention. The relatively slight puffing action of androgen which is reflected in the increase in wet weight of the cloaca is not accompanied by a statistically significant increase in percentage of water. It would appear, therefore, that androgen may not influence the water retention qualities of the cloacal tissues. At any rate there is no evidence of synergism between the 2 hormones in their action on cloacal water retention.

In the comb the growth induced by testosterone propionate was accompanied by a large increase in percentage of water in both breeds; there was very little or no increase in the estrogen-treated lot. The group receiving the mixed hormones showed an increase equal to that in the group receiving androgen alone. Again there was no evidence of either synergism or antagonism.

Figure 2 shows the increase in wet weight plotted as percentage of the control values for both the cloaca and comb of each breed-group. In every case the increase in wet weight is immediately followed by the increase in amount of dry matter and of moisture, each expressed as a percentage of the control lot. This method of illustration

shows that an increase in the wet weight of an organ was due in the main, to an increase in the relative amount of moisture; although at the same time smaller increases in the dry weight did occur.

DISCUSSION

Unpublished data from this laboratory show that estradiol dipropionate produces the same striking effect on the cloaca of the female chick as is described in this paper for the male. In the female the cloacal enlargement is accompanied by a corresponding development of the oviduct. In the female, the cloaca constitutes a common outlet for both intestinal and reproductive tracts, and it is a well known fact that the size of the cloacal outlet, commonly called the vent, is an almost infallible indication of the laying condition of a hen. It appears logical to regard the cloaca as well as the oviduct as an

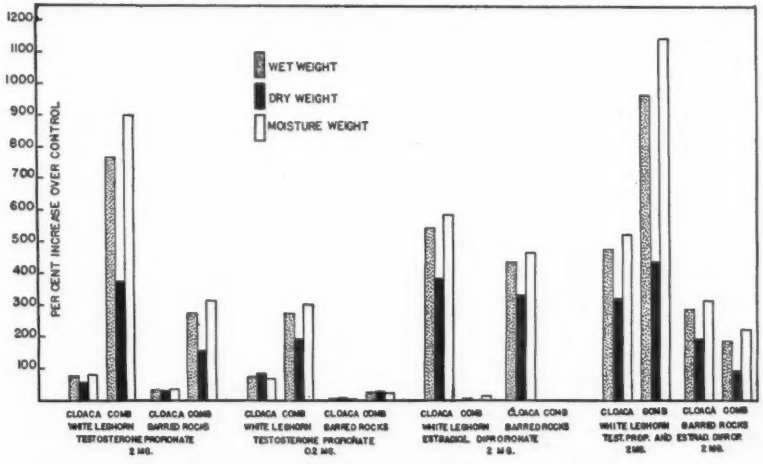

Fig. 2. INCREASE IN WET WEIGHT, DRY WEIGHT AND MOISTURE WEIGHT EXPRESSED IN TERMS OF THE CONTROL. This clearly shows that the increases which occurred in wet weight was mostly due to moisture. It also illustrates the breed difference in responsiveness.

accessory sex organ in this species. The cloaca which is in reality the outlet for the oviduct may, therefore, be regarded as an accessory sex organ common to both sexes and it is not surprising to find both male and female cloacae equally responsive to estrogen.

It would appear to be justified to point out also the analogy between the tissues surrounding the cloacal outlet (i.e., the area in which the puffing reaction occurs) and the sex skin of monkeys. As is the case with the skin surrounding the vent in the chicken, the so-called sex skin of these mammals responds in a minor degree to parenterally administered testosterone propionate but shows a major reaction to estrogen (3). Also, the outstanding characteristic of the androgen-stimulated sex skin in the monkey is hyperemia rather than hypertrophy, which corresponds to the reaction observed in the epidermis of the cloacal region following androgen treatment in this study. Furthermore, it has been shown (5) that the periodic swelling of the sex skin of the baboon is largely due to the accumulation of water and in this respect it also appears to resemble growth of the cloacal region of the chick.

The fact that no evidence for either antagonism or synergism between estrogens and androgens was found in this study is, perhaps, only to be expected. The action

of the hormones on their respective tissues is probably direct because the birds were not sufficiently old to have functional testes whose hormone output could have been decreased by estrogenic depression of the pituitary.

A point which should be mentioned is the breed difference in hormone responsiveness. In all respects the White Leghorns show a greater degree of response than do Barred Rocks. This is probably due to a genetically determined lower response threshold.

SUMMARY

Estradiol dipropionate, in effective doses, produced a 'puffing' reaction in the cloacal region of male baby chicks as early as 48 hours after the first injection. This reaction was progressive under continued treatment until the cloaca resembled that of a laying hen. Testosterone propionate produced an initial 'puffing' reaction accompanied by hyperemia but the cloaca did not show the same progressive enlargement.

The estrogen-treated chicks showed a very extensive enlargement of the sex eminence. The same weight dose of androgen also produced greatly enlarged but comparatively smaller eminences.

The combs were tremendously stimulated by testosterone propionate but estradiol dipropionate produced no measurable increase in comb weight.

The stimulation of combs and cloacae was accompanied by a sharp rise in water content of the tissues.

There was no marked evidence of either synergism or antagonism of action between the male and female hormones used in this study.

The Barred Rocks used in this experiment showed a much smaller degree of response in all respects than did the White Leghorns.

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THE ACID PHOSPHATASE ACTIVITY OF HUMAN URINE, AN INDEX OF PROSTATIC SECRETION[1]

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THE PRESENT INVESTIGATION WAS CONDUCTED to determine, *a*) the normal values for urinary acid phosphatase in men, women and children; *b*) the site of origin of urinary phosphatase; and *c*) the influence of altered physiological states on urinary phosphatase excretion.

That the urine of humans contains an enzyme capable of splitting organic phosphates has been demonstrated by Demuth (1) and others (2, 3, 4). These authors agree that optimal phosphate splitting in urine occurs in an acid medium close to a pH of 5. Kutscher (4) found an alkaline phosphatase present at plasma level in the urine. Considerable controversy exists, however, as to the site of formation of urinary phosphatase, its levels of excretion and sex differences in excretion levels. Waldschmidt-Leitz and Nonnenbruch (2) found a phosphatase present in urine with an optimum pH range of 5 to 6 and in concentrations much stronger than in serum. They believed that the high urinary values were the result of the breakdown of erythrocytes. Dmochowski and Assenhajm (3) believed that urinary phosphatase came from the kidney and did not result from red blood cell destruction.

Wolbergs (5) who found a constancy of urinary acid phosphatase in the fasting human, showed a reciprocal relationship of urinary phosphatase to altered blood sugar levels produced by glucose ingestion or by insulin administration. Kutscher and Wolbergs (6), investigating levels of phosphatase in human prostatic fluid and ejaculates, discovered a very powerful phosphatase whose optimum at pH 5 was in agreement with the urinary phosphatase. They postulated, because of the high concentration in prostatic fluid, that a small amount of this secretion could explain the concentration in the urine.

Demuth (1) found no difference in the acid phosphatase activity in the urine of children and adults. Kutscher and Wolbergs (6) reported an absence of acid phosphatase in the urine of women and old people; one year later, however, Wolbergs (5) showed that the urine of adult women contained ⅓ to ⅕ the phosphatase activity of the urine of adult men.

METHODS

Acid and alkaline phosphatase determinations were made on urine samples according to the method of King and Armstrong, using 0.005 M disodium monophenyl phosphate as a substrate (8); all results are expressed in units as defined by King and Armstrong. The system was buffered at pH 5 and pH 9.3 with 0.2 N sodium acetate-acetic acid and 0.05 M sodium barbital respectively. Samples of 0.5 cc. were used, the urine having first been dialyzed against distilled water for 12 hours in cellophane

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bags. It was found that this period of dialysis was sufficient to eliminate the interference of chromogenic substances. The samples were incubated with the buffer-substrate solution for a period of ½ hour at 37° C. and the colorimetric determinations were made with an Evelyn photoelectric colorimeter using a 6,600 Å filter.

A total of 2,300 determinations was made on 125 individuals. These subjects ranged in age from 2 days to 82 years, and in condition from healthy to pathologic. Both sexes were studied. The pathologic group included a young castrate, two eunochoids, and 25 men who were castrated because of carcinoma of the prostate. At the outset, determinations were made on random, single samples, but later the work was

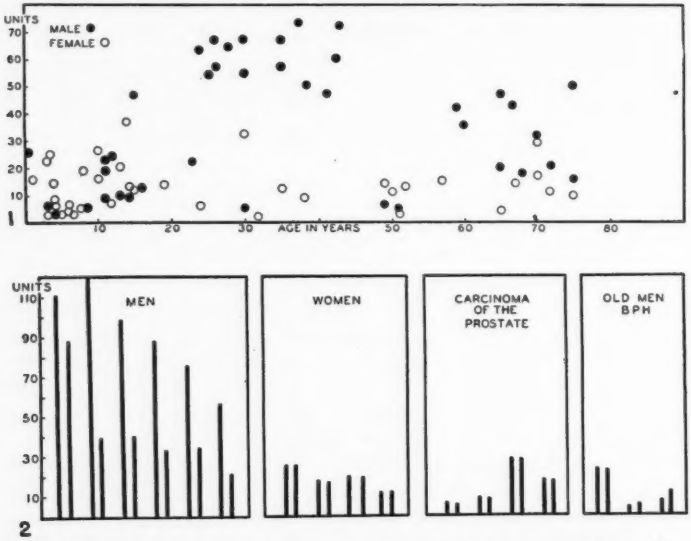

Fig. 1. Average values for acid phosphatase activity in single specimens of urine from normal males and females, ranging in age from 2 days to 75 years. King and Armstrong units per 100 cc. Fig. 2. Acid phosphatase activity in the urine collected according to the two-glass test in normal adult males and females, and in old men with hypertrophy and cancer of the prostate gland. The values are represented in vertical paired bars; in each pair the bar on the left represents the initial specimen and its mate the final urine voided.

expanded and values were determined for a number of different types of collection including *a*) samples obtained before and after coitus, *b*) samples after diuresis, *c*) hourly samples, *d*) ureteral urines obtained at cystoscopy, *e*) total 24-hour specimens of urine and 'two-glass' tests. The two-glass test consisted of collecting the first 30 cc. of urine voided separately from the subsequent urine.

RESULTS

The results for the determination of alkaline phosphatase activity in the urine are in agreement with those of workers who found only traces of this enzyme present.

Values of acid phosphatase expressed in units per 100 cc., for single, random samples without reference to the time of day, food or fluid intake, are shown in figure 1. The phosphatase activity of random samples from normal individuals (table 1) demonstrate the trend shown for 24-hour excertion of the enzyme; namely, that normal young adult males consistently excrete more acid phosphatase in the urine than women of similar age. There is no great difference in the excretion levels

in males or females above 70 years and below 13 years. One newborn male eliminated 25 units per 100 cc. of urine.

Values for 'two-glass' tests (fig. 2) show that only in the male, and particularly normal young adult males, is there a consistently higher value for the first ounce of urine voided than for the rest of the specimen. Females, as well as young boys and old men, failed to show any difference in the first and last urines of the 'two-glass' test. By having a subject void into a series of 5 containers it was determined that the maximum acid phosphatase activity was contained in the first voided sample of 30 cc. of urine; the subsequent samples had identical values which were lower than those of the initial specimen.

TABLE 1. AVERAGE VALUES FOR PHOSPHATASES EXCRETED IN SINGLE SAMPLES OF URINE OF 125 SUBJECTS
The values are expressed in King and Armstrong units per 100 cc.

	King and Armstrong Units
ALKALINE PHOSPHATASE: both sexes	0 to 1.5
ACID PHOSPHATASE	
FEMALES, *age*, yr.	
0–13	13
13–20	12
21–45	20
more than 45	12
MALES, *age*, yr.	
0–13	9
13–20	20
21–50	53
more than 50	27
URETERAL URINES	7
CARCINOMA OF PROSTATE	
Before orchidectomy	
5 subjects	26
After orchidectomy	
25 subjects	18

A striking increase in the enzyme content of the urine passed immediately after ejaculation was noted in 5 subjects. Values as high as 300 units per 100 cc. were obtained on the first ounce of urine voided. However, after the passage of the first 30 cc., subsequent samples of 20 to 30 cc. collected at the same time showed values of 40 to 50 units, which are well within the range of this group. This indicates that seminal fluid did not regurgitate into the bladder at coitus.

The enzyme activity was determined on 19 samples of ureteral urine from 10 patients. Although in one case a high value was obtained, the levels averaged 7.0 units per 100 cc., some 30 units below the value for single, voided specimens in the male and 5 to 10 units below the values for the female. Urines obtained by catheter from the bladder in 7 of these 10 patients showed acid values close to those for ureteral urines. The remaining 3 cases were men, in whom the high phosphatase values for bladder urine may be explained on the basis that prostatic fluid was carried into the bladder with the passage of the catheter.

Urinary acid phosphatase determinations were made preoperatively and postoperatively in a male, age 64, who had a total perineal prostatectomy for an early carcinoma of the prostate (fig. 3). Postoperatively, the enzyme level fell and since has remained at about 10 units per 100 cc. A value of 5 units was determined 4 months after operation.

Excretion values for urinary acid phosphatase in terms of total daily urine output give a more uniform picture than single random samples because variations in the time of day and the fluid and food intake may introduce appreciable errors in the lat-

ter. Seven normal individuals were followed for periods of one month or more, the levels being expressed in terms of units of enzyme excreted in the urine per 24 hours (fig. 4). The urine was collected for the entire 30-day period. The amount excreted

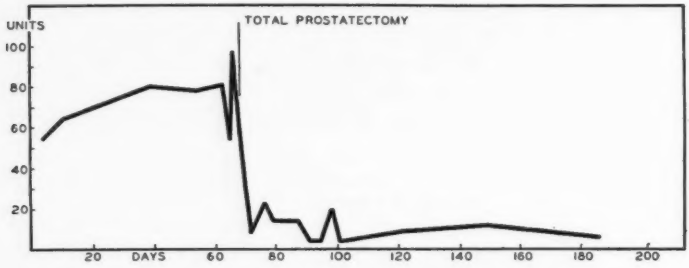

Fig. 3. PREOPERATIVE AND POSTOPERATIVE ACID PHOSPHATASE ACTIVITY in urine of a man with cancer of the prostate in whom total prostatectomy was performed. The values are expressed per 100 cc. of urine.

in the male (fig. 4B) was found to be almost 4 times as much in the female (fig. 4A). The acid phosphatase excretion of the adult males averaged 613 units per 24 hours; the two adult females averaged 163 units per 24 hours. It was of interest to find that the daily output of phosphatase in both adult males and females was nearly constant although at different levels. There was no suggestion of a cycle in any of these adults nor in the male and female children whose daily excretion level is illustrated in figure 5. The average value for the boy, age 8 years, was 48 units per 24 hours (fig. 5A); the girl, age 8 years, excreted an average of 74 units (fig. 5B).

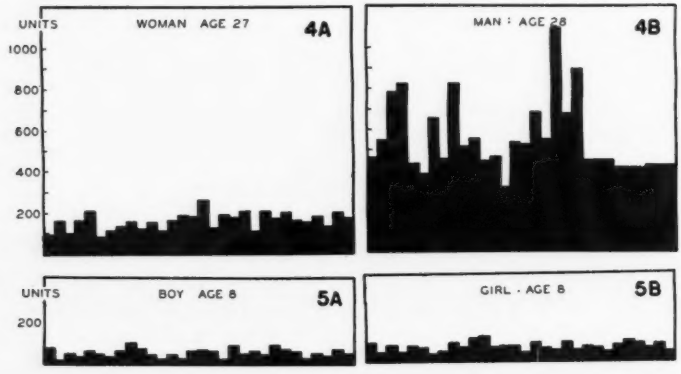

Fig. 4, A and B. DAILY PHOSPHATASE EXCRETION IN ALL OF THE URINE OF a normal adult male and female in a period of 30 days. The values are units excreted in each 24-hour specimen. Fig. 5, A and B. DAILY PHOSPHATASE EXCRETION IN ALL OF THE URINE OF A NORMAL BOY AND GIRL IN A 30-DAY PERIOD.

The 25 men who had advanced carcinoma of the prostate are a part of a series which have had bilateral orchidectomy treatment of their neoplastic disease. Their clinical condition preoperatively and postoperatively and their laboratory records including serum phosphatase determinations, have been described in several earlier publications (9, 10, 11). The total output of acid phosphatase in the urine was de-

termined for 5 of this series for a period of 6 days preoperatively and for 1 to 2 months postoperatively. Before castration, 2 of these patients showed high concentrations of the urinyry enzyme with values of 35 to 70 units per 100 cc. of urine and 250 to 400 units excreted per 24 hours. Preoperatively, the remaining 3 had values of from 15 to 30 units per 100 cc. and 200 to 250 units per 24 hours. Following bilateral orchidectomy, the 2 patients with the high preoperative levels showed a marked decrease in urinary phosphatase in the course of 3 weeks, having values of 10 to 15 units per 100 cc. and 100 to 110 units per 24 hours. The fall in enzyme per 100 cc. was greater than for the total output, because in many of the preoperative determinations the volume of urine produced daily was low. These values following castration are of the same order as those mentioned above for the excretion of the enzyme by women. Two of the remaining 3 patients having relatively low values prior to operation failed to show any postoperative decline. The urinary phosphatase of the third patient fell from a preoperative level of 19 units per 100 cc. and 250 units per 24 hours to 5 units per 100 cc. and 140 units per 24 hours. This decrease was progressive over a period of 25 days.

Little information was derived from the study of the remaining 20 patients with carcinoma becuase urinary phosphatase determinations were made only after operation and only on single samples. However, the average value for all patients was 18 units per 100 cc. These values were determined at a period of from 1 week to as long as 19 months after castration and are considerably lower than most of the preoperative values on the 5 men discussed previously.

The young castrate studied was 30 years of age and been castrated elsewhere at the age of 19 years. When he was first seen he had had treatment with testosterone propionate for several years except for a period of 2 months prior to the first determination of urinary phosphatase. At that time his enzyme level averaged 5 units per 100 cc. urine, and 100 to 150 units per 24 hours, the patient producing large quantities of urine daily (2,000 to 3,000 cc.). It was possible to increase the excretion level to a maximum of 15 units per 100 cc. by the administration of 25 mg. of testosterone propionate per day for a period of 3 weeks.

The first eunuchoid type studied was a man, aged 24 years, with bilateral cryptorchidism; the second was a man of 28 years with moderate atrophy of both testes. Both patients when first seen had been on extended androgen therapy. Urinary phosphatase values for both were erratic and never as low as those for the young castrate, even after administration of testosterone propionate had been discontinued for periods as long as 1 month. As evidenced by the presence of a fairly well developed prostate in each before the administration of androgen these men were not without an endogenous source of androgen.

DISCUSSION

These findings coupled with those of others cited earlier establish clearly that the high values of acid phosphatase in the urine of adult males can be explained on the basis of admixture of prostatic fluid, Adult prostatic epithelium, according to Kutscher and Wolbergs (6) contains large amounts of acid phosphatase; prostatic fluid in man is rich in acid phosphatase; ureteral urine has low activity; voided urine in the adult male has a high acid phosphatase content; removal of the prostate in the adult causes the enzyme content of the urine to fall to low levels which are of the same order as ureteral urines. Granting that most of the increased phosphatase content of urine in normal young males stems from the prostate gland, the nearly constant values found from day to day are evidence in favor of an insensible secretion of the prostate which is likewise nearly constant.

A wealth of evidence exists for the dependence of secretory prostatic epithelium on androgen stimulation. Because of this dependency and our observations establishing the prostate as the site of high urinary acid phosphatase values, we believe that the determination of the urinary enzyme may serve as an index of androgenic activity in the male as well as an index of prostatic secretion. In support of this, reference is made to *a*) the lowering of urinary acid phosphatase in the castrate and *b*) to the 24-hour values showing that during the periods of life when urinary androgen levels are low, as prepuberally and in old men, the urinary acid phosphatase values are low.

Such a relationship is masked at such times as the prostate secretes large amounts of fluid rich in the enzyme. Specifically, high values are obtained after ejaculation. Hence, a male with a low average value for urinary phosphatase at times shows an occasional high value because of admixture of prostatic fluid. However, repeated values over the course of a few days rules out this confusing factor.

SUMMARY AND CONCLUSIONS

The urine of normal adult males contains 3 to 5-fold greater amounts of acid phosphatase than that of normal adult females. There is no great difference in the activity of this enzyme in the urines of males and females below 12 years of age or in old age. Only traces of alkaline phosphatase are excreted in the urine.

The daily output of acid phosphatase in a single individual or in the various age groups is nearly constant, although higher values are obtained following ejaculation. There is no evidence of a cycle in urinary phosphatase excretion.

In the 'two-glass test' in young adult males there is a consistently higher phosphatase activity in the first ounce of urine voided than in the urine which follows; the difference is striking after ejaculation, indicating that semen does not regurgitate into the bladder. Urine obtained from the kidney by ureteral catheter contained small amounts of phosphatase.

In carcinoma of the prostate acid phosphatase values in the urine may be high or low depending in part on the amount of normal prostatic tissue uninvolved by the cancer. Marked decreases in those patients with originally high values followed castration. Following total excision of the prostate a great decrease occurred in urinary phosphatase excretion.

Evidence is presented to support the conclusion that high urinary acid phosphatase values depend in large part upon an admixture of prostatic fluid. Urinary acid phosphatase, therefore, is an index of prostatic secretion; much of this secretion is insensible and occurs at a nearly uniform rate. Because of the dependency of prostatic epithelium upon androgens, the measurement of urinary acid phosphatase is suggested as an index of activity of the prostate gland and as a crude index of androgenic activity in the male.

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BLOOD-SUGAR LEVELS AND CARBOHYDRATE COMBUSTION IN NORMAL MEN[1]

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IN A PRELIMINARY REPORT (1) we expressed the view that the level, per se, of the blood sugar is not the determining factor in the regulation of carbohydrate oxition in the intact organism; that hyperglycemia associated with the addition to the body of carbohydrate from outside sources, produces under standard conditions a rise in the proportion of energy derived from carbohydrate; that hyperglycemia of wholly endogenous origin produced by the administration of epinephrine, results in no significant change in the rate of combustion of carbohydrate; and, finally, that the rise of the blood sugar resulting from the administration of carbohydrate is merely concomitant to those stimulative processes which are initiated when the carbohydrate reserves of the body are increased. The details of our experiments are herein recorded.

METHODS

Detailed observations were made on 3 healthy, male, university students, ranging in age from 21 to 24 years. Twenty-one 4-hour periods of continuous indirect calorimetry by the open-circuit method (2) were performed on these subjects, a large respiration chamber being employed for this purpose. Preparatory to each experiment and for a minimum of 5 days a constant diet was eaten by each subject. It consisted of 80 gm. of protein, 100 gm. of carbohydrate and sufficient calories for maintenance of body weight on full activity. Calories ranged from 3400 to 3800 daily. Activity from day to day was made as routine and constant as possible. The preparatory diet was relatively low in its content of carbohydrate because it was felt that under these conditions small additions or subtractions of carbohydrate to and from the body stores would reflect greater changes in metabolism than would have occurred had the body reserves of carbohydrate been large.

All calorimetric experiments were performed approximately from 8:00 A.M. to noon. The subjects were thus studied *a*), in the postabsorptive state, *b*), in the postabsorptive state plus the subcutaneous injection of 1 mg. of epinephrine and *c*), after the ingestion of 60 gm. of dextrose dissolved in 300 cc. of water. Under precisely the same conditions blood-sugar curves were obtained. In a fourth set of experiments similar observations were made to note the effects of a combination of injection of epinephrine and ingestion of dextrose.

On the evening preceding an experiment the trained subject was put to bed in the chamber. The chamber was then sealed and the entire mechanism set into opera-

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TABLE 1. COMBUSTION OF CARBOHYDRATE

Experiment No.	Procedure		Heat Production cal.	CHO Oxidized gm.	% of Total Cal. Derived from CHO
	Subject L.L., 3600 Calories				
1	Fasting		336	23.1	25.0
2	Fasting		335	23.4	26.1
		Average	336	23.3	25.6
3	Dextrose, 60 gm. orally		354	49.3	52.0
4	Dextrose, 60 gm. orally		301	40.2	50.0
		Average	328	44.8	51.0
5	Epinephrine, 1 mg. subcut.		395	19.2	18.0
6	Epinephrine, 1 mg. subcut.		385	21.5	21.0
7	Epinephrine, 1 mg. subcut.		371	26.1	26.3
		Average	384	22.3	21.8
	Subject B.S., 3800 calories				
1	Fasting		314	26.7	31.9
2	Dextrose, 60 gm. orally		322	40.7	46.6
3	Dextrose, 60 gm. orally		310	32.1	38.7
		Average	316	36.1	42.7
4	Epinephrine, 1 mg. subcut.		372	18.3	18.4
	Subject A.C., 3400 calories				
1	Fasting		328	18.7	21.3
2	Fasting		337	27.3	28.0
3	Fasting		334	26.3	29.4
		Average	333	24.1	26.2
4	Dextrose, 60 gm. orally		379	40.2	39.6
5	Dextrose, 60 gm. orally		327	30.6	35.0
6	Dextrose, 60 gm. orally		356	29.1	30.6
		Average	354	33.3	35.1
7	Epinephrine, 1 mg. subcut.		367	21.8	22.2
8	Epinephrine, 1 mg. subcut.		402	23.1	21.5
		Average	385	22.5	21.9
9	Dextrose, 60 gm. orally Epinephrine, 1 mg. subcut.		394	35.5	33.7
10	Dextrose, 60 gm. orally Epinephrine, 1 mg. subcut.		395	33.3	31.5
		Average	395	34.4	32.6
	Subject A.S., 2600 calories				
1[2]	Fasting		253	14.3	20.6
2	Fasting		257	17.7	25.7
3	Dextrose, 60 gm. orally		267	40.8	57.1
4	Epinephrine, 1 mg. subcut.		279	16.2	21.8

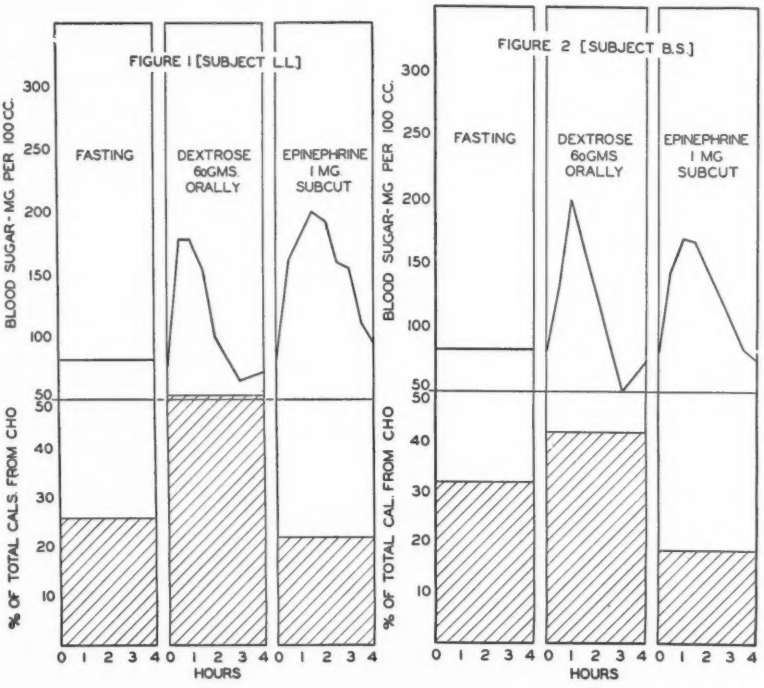
[1] Four-hour test periods in respiration chamber. Preparatory diet: protein 80 gm., carbohydrate, 100 gm. Analytical data in table 5.

[2] Hypoglycemic attack with trembling during last 7 minutes of period. Experiment stopped at 3½ hours.

tion. At 8:00 o'clock the next morning the turning of 2 stopcocks marked the beginning of the collection of a continuous sample of chamber air. Analysis of gases was done by the technic of Carpenter (3). Calculation of respiratory data was made in the standard way (4). Blood sugar was determined by the Benedict method (5) and urinary nitrogen by the Kjeldahl method.

RESULTS

The respiratory data obtained on *subject L.L.* is presented in table 1. In the postabsorptive state he derived 25.6% of his total heat production from the combustion



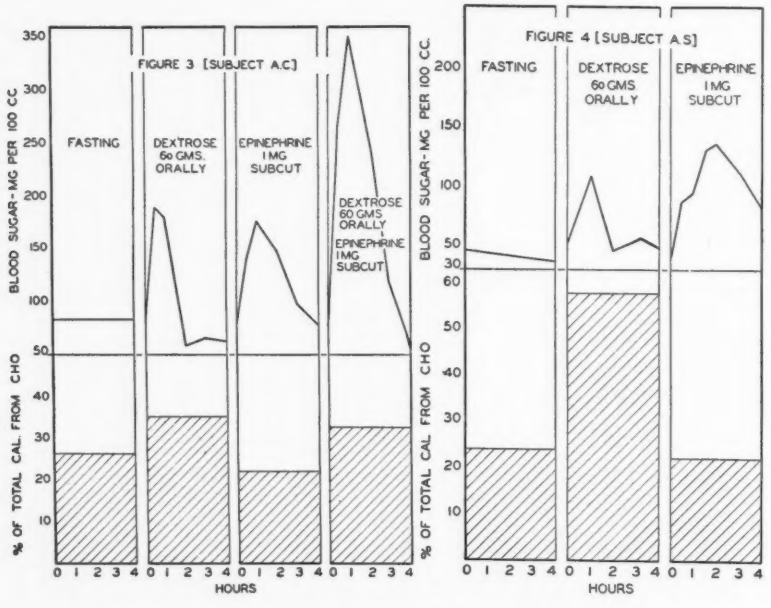
of carbohydrate. The ingestion of 60 gm. of dextrose resulted in an increase in the combustion of carbohydrate to the extent of 51% of the total energy exchange for the period. The subcutaneous administration of 1 mg. of epinephrine was associated with no increase in the total amount of carbohydrate oxidized, over that observed in the postabsorptive state, despite a 14% increase in total heat production, which increment was supplied wholly by increased combustion of fat. The proportion of total energy contributed by the combustion of carbohydrate fell, therefore, to 21.8% as compared with 25.6% in the fasting state without epinephrine.

Figure 1 shows graphically the lack of relationship between the level of the blood sugar and the degree of combustion of carbohydrate. Despite the plethora of circulating blood sugar in the case of epinephrine hyperglycemia as compared with postabsorptive values of blood sugar, there occurs a slight diminution, below the postabsorptive value, in the capacity of the subject to oxidize carbohydrate. The addition

of extra carbohydrate to the body, on the other hand, which, to be sure, is associated with an absorptive rise of the blood sugar level, results in a marked increase in the proportion of energy derived from the oxidation of carbohydrate.

Subject B.S., (table 1, fig. 2) gave essentially the same results. The ingestion of dextrose produced hyperglycemia and a significant rise in the combustion of carbohydrate as compared with the value obtained in the postaborptive state. The injection of epinephrine, however, while producing a similar degree of hyperglycemia, and, in addition, an 18% increase in total heat production, resulted in the oxidation of distinctly less carbohydrate than was utilized in the postabsorptive state.

Table 1 and figure 3 summarize the extensive data obtained on *subject* A.C. studied continuously over a period of 4 months. It is to be noted first that precisely the



same results were obtained in this subject as in the 2 previous ones. Again an increase of 18% in the total heat production after the administration of epinephrine was met wholly by an increased oxidation of fat. The percentage of total energy obtained from the combustion of carbohydrate was lower than that observed in the postabsorptive state; and comparable elevations of the blood sugar levels, produced on the one hand by the injection of epinephrine and on the other by ingestion of dextrose resulted in widely different rates of combustion of carbohydrate.

The last 2 experiments on *subject* A.C. dealing with the effects of simultaneously administered dextrose and epinephrine, are of particular interest (last quarter of fig. 3). Note that when 60 gm. of dextrose was ingested, the actual number of grams of carbohydrate oxidized and percentage of total energy derived in this process were essentially the same whether or not the subject received, in addition, 1 mg. of epinephrine subcutaneously. The presence of epinephrine activity has not interfered with the normal stimulation of carbohydrate oxidation, which is invariably produced by the ingestion of 60 gm. of dextrose. Furthermore, a blood sugar level of 350 mg.%

produced by the combined effects of ingestion of dextrose and injection of epinephrine resulted in no greater combustion of carbohydrate than a blood sugar level of 170 mg.% produced by the ingestion of dextrose alone.

It is evident that the rates of combustion of carbohydrate change significantly only in relation to the intake of carbohydrate and regardless of the level of the blood sugar.

DISCUSSION

It has been claimed that utilization of carbohydrate by the 'normal' dog is directly related to the level of the blood sugar (6). The preparation considered normal was the eviscerated animal. The method of changing the blood sugar level was to vary the amount of exogenous carbohydrate administered per unit of time. The rate of utilization of carbohydrate by the animal, therefore, was related to the amount of carbohydrate administered per unit of time. The resulting blood sugar levels were, of course, related to this same factor.

There can be no question about the fact that in the normal organism the rate of utilization of carbohydrate is related directly to the amount of carbohydrate administered (7); but, it does not follow that the level of the blood sugar, thus produced, controls the rate of utilization of carbohydrate. We, therefore, chose to compare the effects of hyperglycemia of wholly endogenous origin, produced by epinephrine, with those resulting when the hyperglycemia is associated with the addition of carbohydrate to the body.

Innumerable studies dealing with the effects of epinephrine upon the metabolism of carbohydrate have been reported. Upon several points there is essential agreement: *a*), that a single injection of epinephrine produces a rise of the blood sugar (8); *b*), that it results in a rise of the blood content of lactic acid with a resultant 'blowing off' (auspumpung) of CO_2 and a fall of the CO_2 combining capacity of the blood (8–11); and *c*), that the blood lactic acid and blood CO_2 combining power return to their normal values in 2 to 3 hours (9, 11). The latter observation is of primary importance in the interpretation of respiratory quotients after the administration of epinephrine. It means that such experiments must be carried out over a sufficiently long period to allow the initial excessive expiration of CO_2 to be compensated by the later retention of CO_2, this exchange having been accomplished when the acid-base equilibrium of the body has returned to its original value. It is important, also, that the method of continuous sampling of respiratory gases be employed so that an over-all determination of the respiratory exchange be possible, since an average respiratory quotient based on frequent short periods of sampling may be very misleading under the circumstances. The method used in our experiments (four-hour periods of continuous indirect calorimetry) satisfies these requirements.

It is with regard to the specific effect of epinephrine upon the total combustion of carbohydrate by the normal organism that much disagreement exists. Depending upon the method of investigation and the experimental preparation used, results in animals have indicated *a*), that epinephrine does not affect the utilization of carbohydrate by the tissues (12, 13), *b*) that it is capable of suppressing completely the oxidation of carbohydrate in the organism (14, 15, 16), and *c*), that it actually increases the rate of utilization of carbohydrate by the tissues (17). Our results, in this respect, agree with those of Courtice et al. (11) and Dill et al. (18) that in the normal, fasting human being at rest, a single injection of epinephrine does not affect significantly the proportion of total energy derived from carbohydrate. If, at all affected, the rate of combustion of carbohydrate is somewhat diminished. Our results indicate, also, that epinephrine does not influence the proportion of total energy derived from carbohydrate in the dextrose fed, resting subject as well (table 1 and fig. 3).

The presence or absence of a relationship between the blood-sugar level, per se, and the rate of combustion of carbohydrate is obviously important with regard to present day theories of diabetes mellitus, in particular, and carbohydrate metabolism in general. That epinephrine, given in sufficient quantity to produce prolonged hyperglycemia in the normal fasting subject, results in no increase in the combustion of carbohydrate over that observed in the fasting state at its usual level of blood sugar, is a clear conclusion from our results. Cori (19), while in complete accord with these

TABLE 2. ANALYTICAL DATA

Subject	Experiment No.	CO_2 Eliminated	O_2 Absorbed	Urinary Nitrogen	Total R.Q.	Non-protein R.Q.
		liters	*liters*	*gm.*		
L.L.	1	56.21	70.96	1.83	.792	.790
	2	55.85	70.57	1.59	.791	.790
	3	63.57	73.29	1.86	.867	.879
	4	53.80	62.50	1.57	.861	.871
	5	64.08	83.56	1.55	.767	.763
	6	53.06	81.41	1.48	.775	.771
	7	65.72	83.47	1.82	.787	.785
B.S.	1	53.52	66.08	1.90	.810	.813
	2	57.19	67.01	2.00	.853	.865
	3	53.95	64.86	1.96	.832	.838
	4	60.82	78.85	1.95	.771	.766
A.C.	1	54.44	69.60	2.10	.782	.778
	2	56.80	71.11	2.09	.799	.798
	3	56.61	70.35	2.15	.805	.806
	4	65.94	79.28	2.03	.832	.837
	5	56.37	68.73	2.01	.820	.824
	6	60.62	74.97	2.29	.809	.810
	7	60.80	77.58	2.12	.784	.780
	8	66.60	85.13	2.42	.782	.778
	9	67.62	82.79	2.45	.816	.820
	10	67.28	83.15	2.29	.809	.811
A.S.	1[1]	41.84	53.61	1.47	.780	.776
	2	43.06	54.26	1.55	.794	.792
	3	48.64	55.02	1.58	.884	.901
	4	45.99	58.92	1.46	.781	.777

[1] 3½ hour experiment.

results, believes that the increase in sugar oxidation, which would otherwise be associated with an increase of the blood sugar level, is prevented by an action of epinephrine at the peripheral tissues. If this were so, under the conditions of our experiments, the normal course of oxidation of ingested carbohydrate should be impaired by the simultaneous action of epinephrine. As was seen in table 1 and figure 3, however, epinephrine in no way interfered with the normal course of combustion of ingested carbohydrate. Yet a blood sugar level of 175 mg.% produced by epinephrine alone provoked no increase in the oxidation of carbohydrate. Further, essentially the same degree of combustion of carbohydrate was obtained as a result of the ingestion of 60 gm. of dextrose whether or not epinephrine was acting simultaneously. And finally, the degree of combustion of carbohydrate resulting from the ingestion of 60 gm. of dextrose was independent of the level of the blood sugar (190 mg.% as compared with 350 mg.%).

A separate point should be made, however, with regard to Cori's contention. The evidence that prolonged and continuous administration of epinephrine leads to a markedly diminished capacity of the organism to oxidize blood sugar appears definite

(14, 15, 16). This does not necessarily imply a direct effect of epinephrine on the peripheral oxidative mechanism. An indirect effect, based upon a gradual depletion of the carbohydrate reserves of the body could likewise be assumed. Indeed, this appears to have been the state of affairs obtained in the experiments of Bridge and Noltie (20) and Samson (21). For our purpose, that of demonstrating the lack of relationship between the blood sugar level and the rate of combustion of carbohydrate, a small quantity of epinephrine, sufficient only to produce hyperglycemia for 3 to 4 hours, was considered adequate.

We wish, at this point, to refer briefly to corroborative evidence of a practical nature, which we have recently reported in detail elsewhere (22). It concerns studies, similar to those described here, upon a patient suffering from spontaneous hypoglycemia, the latter subsequently proven at operation to be due to several islet cell tumors of the pancreas. Table 1 and figure 4, reconstructed from previous data in order to be similar in form to the others reported here, show some of the preoperative results. It is seen that the ingestion of 60 gm. of dextrose produced a supernormal increase in the rate of combustion of carbohydrate. This was the invariable result of the addition of carbohydrate to the body, under all of a variety of conditions. In this particular experiment the marked increase in combustion of carbohydrate was accompanied by a rise of the blood sugar from 50 mg.% to 108 mg.%. If this small rise of blood sugar which accompanied such a marked increase in the rate of oxidation of carbohydrate, indicated that this patient was particularly sensitive to elevations of the blood sugar, epinephrine hyperglycemia should precipitate a similar response; but, despite an elevation of the blood sugar level from 40 mg.% to 132 mg.% produced by epinephrine, no increase in the combustion of carbohydrate over that found in the fasting state was observed. Attempts to control the hypoglycemia by administration of carbohydrate, on the other hand, resulted in excessive oxidation of carbohydrate.[2]

It is not implied that dextrose of endogenous origin is treated in a different manner by the tissues than dextrose of exogenous origin. It is suggested, however, that in the intact animal the tissues are conditioned with regard to their ability to oxidize carbohydrate, by the level of carbohydrate reserves of the body, rather than by the amount of sugar circulating in the blood; that an increase in the reserves of carbohydrate initiates a mechanism which increases the rate of combustion of carbohydrate by the body; that those procedures which decrease the reserves of carbohydrate in the body tend to inhibit oxidation of carbohydrate by the tissues; and that these mechanisms are independent of the level of circulating blood sugar.

Experiments on intact human beings, such as are here reported, do not permit analysis of intermediary processes such as can be obtained by the removal of various organs in the experimental animal. They do, however, allow a final evaluation of the resultant effect of a given procedure upon the human body in a state of normal physiological function. It has been our desire to avoid the technic of removal of vital organs, especially when the results are to be interpreted in terms of normal physiological function.

CONCLUSIONS

In normal, fasting men a prolonged elevation of the blood sugar level, produced by epinephrine, is accompanied by no greater combustion of carbohydrate than is obtained at the usual fasting blood sugar level.

Following the ingestion of a given amount of dextrose there occurs an increase in the rate of combustion of carbohydrate which is the same whether or not epinephrine

[2] Epinephrine, therefore, is a useful therapeutic measure in such cases since it produces an elevation of the blood sugar without, at the same time, stimulating increased combustion of carbohydrate.

is administered with the dextrose; this, despite the fact that the resulting blood sugar levels are widely different.

With the same degree of hyperglycemia, approximately twice as much carbohydrate is oxidized when the hyperglycemia is produced by the ingestion of dextrose as when it is produced by the administration of epinephrine.

The level, per se, of the blood sugar does not control the rate of combustion of carbohydrate in normal men.

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THE EFFECT OF INJECTION OF A SALINE EXTRACT OF ANTERIOR PITUITARY ON THE GLUCOSE TOLERANCE OF RATS

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PERMANENT DIABETES has been produced in the dog by means of the injection of suitable extracts of the anterior pituitary by a number of investigators (1–4). However, as yet no one has been able to obtain similar results in the intact rat. Permanent diabetes has been established in this species by the injection of anterior pituitary extracts only after partial pancreatectomy (5). This resistance of the rat to anterior pituitary extract has attracted considerable attention. Anselmino, Herold, and Hoffmann (6) reported that the administration of an aqueous extract of an acetone-dried pituitary preparation to normal rats resulted in a substantial increase in the number and size of the islets of Langerhans in a few days. Hoffmann and Anselmino (7) showed that these histological changes were associated with a fall in blood sugar and attributed these effects to a pancreatropic action of the pituitary extract. In 1937 Richardson and Young (8) using a quantitative method for evaluation of the ratio (islet tissue/acinar tissue) for the pancreas of the rat, reported that the mean value of this ratio in rats injected with saline extracts of fresh pituitary gland for a period of 1 to 2 weeks was double that in the controls. They were, however, unable to confirm the results of the German investigators with the type of extract used by the latter.

In 1939 Marks and Young (9) found that the insulin content of the pancreas of rats treated with an extract of anterior pituitary was increased to more than twice that of control animals. The same extract injected into dogs resulted in a decrease in the insulin content of the pancreas. This evidence suggested that the resistance of the rat to the diabetogenic action of anterior pituitary extracts might be due to compensatory hyperplasia of the islets of Langerhans with increased insulin production.

Richardson and Young (8) reported no significant change in the blood sugar of rats treated with extracts of the anterior pituitary despite the histological evidence of hyperplasia of the islets of Langerhans. However, in a group of one-month-old rats injected with saline anterior pituitary extract the blood sugars in the postabsorptive state were found by us to be consistently lower than those in control animals. The determination of the glucose tolerance of animals treated with anterior pituitary extracts was therefore undertaken to see whether changes in carbohydrate tolerance occur which could be related to the pancreatic changes described above and which might throw additional light on the resistance of the rat to the diabetogenic action of extracts of the anterior pituitary.

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EXPERIMENTAL

Rats of the Connecticut Agricultural Experiment Station strain were used in these studies. They were fed a diet of Purina fox chow, ad libitum, and weighed amounts of bread and meat.

A standardized glucose tolerance test was performed following a 24-hour fast. The rats were injected intraperitoneally with 200 mg. of glucose per 100 gm. of body weight as a 5% glucose solution in 0.9% saline. Blood samples were taken after 30, 90, and 180 minutes. The blood was taken from the tail vein after a local hyperemia was produced by heating in warm water. Blood sugar was determined by the Hagedorn Jensen method (10) on a zinc sulfate-sodium hydroxide filtrate (11) using 0.1 or 0.2 cc. of blood.

The rats were injected daily either subcutaneously or intraperitoneally with a crude saline extract of beef anterior pituitary glands, prepared twice weekly. The pituitary glands were frozen in carbon dioxide snow immediately upon removal from the animal at the slaughter house. They were brought to the laboratory in the frozen state. The anterior lobe was carefully dissected from the remainder of the gland and ground in a mortar with sand and ice-cold 0.9% NaCl solution. The insoluble material was removed by centrifugalization. The extract was prepared so that 2.0 cc. of extract were equivalent to 1 gm. of fresh gland.

RESULTS

The results of repeated glucose tolerance tests on untreated rats of various age groups are given in table 1. The mean ± the standard deviation, is given for the

TABLE 1. GLUCOSE TOLERANCE OF UNTREATED RATS AT VARIOUS AGES

No. of rats	No. of tests	Age range	Blood Sugar mg./100 cc.			
			Min. after glucose administration			
			Fasting	30	90	180
		mo.				
10	28	1–2	74±13[1]	147±21	108±11	97±10
6	13	2–3	83± 6	166±28	123± 8	106± 8
3	4	3–4	75	163	118	106
3	3	4–5	81	148	117	107
3	3	7–8	83	146	108	103
5	9	10–12	71± 6	144±14	113± 7	100± 9
	60	Av.	76± 1[2]	152± 3	113± 1	101± 1

[1] Mean±standard deviation.
[2] Mean±standard error of mean.

several age groups in which the number of tests performed was large enough to warrant statistical treatment. The mean ± the standard error of the mean is given for the total group of untreated rats. It is evident that using the standardized tolerance test as described, no significant change in the tolerance to glucose is correlated with age. Glucose tolerance tests were repeated on the same animal at intervals with uniform results. Rats of both sexes were used and no differences were observed in their tolerance to glucose.

Rats of approximately 1 month of age were injected daily with increasing amounts of saline A.P.E. (prepared as previously described), and glucose tolerance tests were repeated at about weekly intervals. The period of injection ranged from 3 to 5 weeks. In tables 2 and 3 are shown the results of such tests.

TABLE 2. EFFECT OF REPEATED INJECTIONS OF A.P.E. ON GLUCOSE TOLERANCE OF YOUNG RATS

Rat no.	Date	Amt. of A.P.E.[1]	Blood Sugar mg./100 cc.			
			Min. after glucose administration			
			Fasting	30	90	180
		gm.				
Average of untreated rats			74±2	147±4	108±2	97±2
16, ♀	1/8	2.5	58	155	75	72
	1/17	11.5	65	99	74	76
	1/24	18.5	62	127	65	88
	1/30	24.5	56	146	74	70
	2/5	31.5	53	162	92	81
18, ♂	1/17	11.5	72	94	81	81
	1/24	18.5	81	127	99	90
	2/1	26.5	60	152	81	81
	2/9	34.5	66	145	86	92
	2/18	52.0	58	113	86	79

[1] Amount of A.P.E. in terms of gm. of fresh gland given up to time of experiment. Injection of A.P.E begun on 1/4.

The individual tolerance tests of 2 rats are given in table 2. For comparison, the mean ± the standard error of the mean for the untreated group of rats of the same age group is shown at the top of the table. In these animals a pronounced effect upon the glucose tolerance curve was produced by the injection of the extract. The nature of the effect, however, is opposite to that which might be anticipated. The curves of the treated rats indicate an apparent increased tolerance to glucose, as shown by the rapid drop of the blood sugar almost to the fasting level 90 minutes after glucose administration. Once this effect appears no further change is found with continued treatment.

In table 3 are given the averages of glucose tolerance tests performed during the period of injection with A.P.E. for all of the 30-day-old rats. The concentration of blood sugar in the fasting state and the rise in blood sugar at the end of 30 minutes following the administration of glucose was approximately the same in both groups. The

TABLE 3. EFFECT OF REPEATED INJECTIONS OF A.P.E. ON GLUCOSE TOLERANCE OF YOUNG RATS[1]

Rat no.	No. of tests	Blood Sugar mg./100 cc.			
		Min. after glucose administration			
		Fasting	30	90	180
1, ♀	2	64	111	73	69
7, ♂	3	80	164	82	72
4, ♀	2	63	137	75	62
13, ♀	4	73	129	88	89
15, ♂	5	73	137	89	87
16, ♀	5	59	138	76	77
17, ♀	3	70	136	94	83
18, ♂	5	67	126	87	85
41, ♂	3	78	179	82	96
Average of treated		70±2	139±5	84±2	82±2
Average of controls		74±2	147±4	108±2	97±2

[1] Injections of A.P.E. begun at approximately one month of age.

TABLE 4. GLUCOSE TOLERANCE OF YOUNG RATS FOLLOWING CESSATION OF TREATMENT WITH A.P.E.

Rat no.	Date	Blood Sugar mg./100 cc.			
		Min. after glucose administration			
		Fasting	30	90	180
1		*27.5 gm. A.P.E. given over period of 29 days*			
	12/16	62	123	82	72
	12/19	*Injection of A.P.E. discontinued*			
	12/23	60	114	95	77
	12/30	59	142	98	79
	1/6	78	142	110	107
	1/14	74	136	120	100
4		*33.0 gm. A.P.E. given over period of 33 days*			
	12/20	65	152	82	63
		Injection of A.P.E. discontinued			
	12/23	60	142	95	83
	12/30	59	142	98	79
	2/3	59	140	123	112

striking change as pointed out above was the extremely rapid fall in blood sugar in the treated animals as compared with the control values. These results were obtained consistently in all young rats treated with A.P.E. When the injection of extract was discontinued after the increased tolerance to glucose had been produced, there was a gradual return of the glucose tolerance to the control level, as shown in table 4.

In table 5 are shown the results of glucose tolerance tests in 30-day-old rats 24 hours following the injection of a single dose of A.P.E., equivalent to 0.5 or 1.0 gm. of fresh gland. It may be seen that with this amount of treatment no marked change in the tolerance curve was apparent. In contrast to these findings are those shown in table 6, in which the glucose tolerance tests of adult rats, which were injected with comparable amounts of extract in proportion to body weight, are given. For each animal the results of a control glucose tolerance test as well as the values following treatment, are tabulated. In many of these animals the glucose tolerance was greatly altered following a single injection of A.P.E. The rise in blood sugar 30 minutes after glucose administration was much greater than in the control tests, and the subsequent fall in blood sugar less rapid. In *rat 34* particularly, the high fasting value, the marked rise following glucose administration, and the slow fall is similar to that which might be expected in a diabetic animal.

When daily injections of A.P.E. in the adult rat were continued, the glucose tolerance curve obtained progressively changed as may be seen in table 7. Although the rise in blood glucose following the glucose injection was sustained, the subsequent

TABLE 5. EFFECT OF SINGLE INJECTION OF A.P.E. ON GLUCOSE TOLERANCE OF YOUNG RATS

Rat no.	Amt. of A.P.E.	Blood Sugar mg./100 cc.			
		Min. after glucose administration			
		Fasting	30	90	180
	gm.				
41	0.5	91	126	109	104
42	0.5		144	94	86
43	1.0	94	120	94	109
46	1.0	79	135	90	90
47	1.0	83	133	109	109

TABLE 6. EFFECT OF SINGLE INJECTION OF A.P.E. ON GLUCOSE TOLERANCE OF ADULT RATS

Rat no.	Age mo.	A.P.E. treatment, gm.	Blood Sugar mg./100 cc.			
			Min. after glucose administration			
			Fasting	30	90	180
37, ♂	7	C[1]	81	166	118	—
		2.5	90	323	147	103
32, ♀	10	C	67	126	116	103
		2.5	82	198	116	107
27, ♀	10	C	70	134	107	99
		2.5	86	232	124	99
31, ♀	10	C	67	155	122	—
		2.5	74	237	175	116
35, ♂	7	C	82	147	127	—
		2.5	95	261	201	120
28, ♀	10	C	74	138	106	113
		5.0	81	365	162	105
34, ♂	7	C	78	123	98	98
		5.0	110	355	308	—
36, ♂	7	C	89	149	107	107
		5.0	91	309	208	116

[1] Control glucose tolerance test done before treatment with A.P.E.

fall was more rapid, so that after 2 weeks of treatment the values obtained at the 90 and 180-minute periods were actually lower than those in the control studies on the same animal. Following cessation of treatment with A.P.E. the glucose tolerance curve gradually returned to normal, as indicated in the studies on *rats 25* and *26*.

DISCUSSION

Crude anterior pituitary extracts have been demonstrated to inhibit insulin action (12) and to depress carbohydrate utilization (13). In the dog and the partially pancreatectomized cat and rat, injection of these extracts results in histological changes in the islets of Langerhans and the appearance of diabetes. It seems surprising, therefore, that in the intact rat these same extracts should produce an increased tolerance to glucose.

The data presented here suggest that the altered glucose tolerance curve obtained in the rats receiving A.P.E. may be the result not only of the direct action of the extract but also of secondary compensatory reactions. This is shown most clearly by the sequence of events in the rats of the older age group. In these animals the first effect observed after a single injection of extract is a diabetic type of glucose tolerance curve. In this connection it is of interest that a temporary glycosuria has been produced in the intact rat by the injection of crude saline A.P.E.; this disappeared with continued treatment (14). As the injections of A.P.E. in the present experiments were continued, the glucose tolerance was markedly altered. The initial rise in concentration of blood glucose following the injection of glucose continued to be higher than normal, but subsequently the blood sugar dropped rapidly so that by the end of 90 minutes the concentration was significantly lower than in the control studies. This peculiar type of response to the injection of glucose might be explained by a combination of two factors, such as an inhibitory effect upon glucose utilization and a second-

TABLE 7. EFFECT OF REPEATED INJECTIONS OF A.P.E. ON GLUCOSE TOLERANCE OF ADULT RATS

Rat no.	Age, mo.	Date	Amt. of A.P.E. gm.	Blood Sugar mg./100 cc.			
				Min. after glucose administration			
				Fasting	30	90	180
10, ♂	4½	3/12	0.0	79	138	110	103
		3/17	10.0	81	212	123	98
		3/24	27.5	67	221	89	83
		3/28	39.5	79	211	102	88
11, ♂	4½	3/12	0.0	83	141	122	109
		3/17	10.0	69	308	189	114
		3/24	27.5	61	261	117	74
		3/28	39.5	82	258	99	78
25, ♂	3½	3/24	0.0	66	126	109	102
		3/28	10.0	85	262	102	89
		4/4	25.0	82	240	94	94
		4/11	52.5	82	275	82	85
			Injections of A.P.E. discontinued				
		4/17		85	251	82	90
		4/23		74	184	98	96
		4/30		67	184	110	100
26, ♂	3½	3/24	0.0	83	192	123	103
		3/28	10.0	70	306	124	109
		4/4	25.0	70	224	90	88
		4/11	52.5	74	280	82	80
			Injections of A.P.E. discontinued				
		4/17		79	248	88	85
		4/23		86	195	106	89
		4/30		70	234	129	90

ary compensatory response of the animal resulting in an increased rate of glucose utilization.

In the young rats the findings are strikingly different from those obtained in the older animals. In these rats no diabetogenic effect was observed at any time during A.P.E. injection, but within a few days after the injection of A.P.E. had been started an increased tolerance for glucose was apparent. This would suggest that these young animals have a much greater capacity for adapting to the inhibitory action of the anterior pituitary extract on carbohydrate utilization.

The exact mechanism whereby the intact rat responds to the injection of A.P.E. by developing an increased tolerance for glucose cannot be directly determined. There is much indirect evidence, however, to suggest that the phenomenon is due to increased insulin production. Histologically, the pancreas tissue of rats which have been treated similarly to these animals show hyperplasia of the islets of Langerhans (8) and assays have revealed increased insulin content of these organs (9). The glucose tolerance curves found in the treated animals might be explained on the basis of increased insulin secretion. If this were the case it would indicate that in the rat the inhibitory action of A.P.E. on carbohydrate utilization can be completely offset by increased insulin production. This could account for the refractoriness of the rat to the diabetogenic action of A.P.E. There is evidence in other species, such as the cat and dog, that insulin protects against the diabetogenic action of A.P.E. (15, 16).

Haist and Best (17) have shown that the insulin content of the pancreas in hypophysectomized rats is slightly less than in normal animals fed ad libitum but is of the same order as that of controls receiving a similar caloric intake. Griffiths and Young

(18) also have reported that removal of the pituitary gland does not result in diminution in size or activity of the pancreas in proportion to body weight. This evidence is against the existence of a pancreatropic hormone, that is, a hormone necessary for the maintenance and function of the pancreatic islets. The increase in amount of islet tissue (8) and of insulin (9) in the pancreases of rats treated with extracts of the anterior pituitary gland, as well as the results of the glucose tolerance tests reported here, may all be secondary to an extra-pancreatic effect of the extract and be compensatory in nature.

The striking difference between the response of the young and adult rat to A.P.E.

TABLE 8. TWO-DOSE GLUCOSE TOLERANCE TEST IN YOUNG AND ADULT UNTREATED RATS[1]

Rat no.	Age mo.	Blood Sugar mg./100 cc.					
		Min. after first dose of glucose					
		Fasting	30	90	120	180	270
44	1½	70	141	112	144	103	100
45	1½	81	155	115	170	134	118
49	1½	67	141	95	141	118	113
50	1½	56	163	106	158	105	107
	Av.	69	150	107	153	115	110
1	6	72	149	93	158	123	120
7	6	78	184		184	143	130
27	11	81	158	109	152	127	131
31	11	67	155	122	166	134	120
32	11	72	138	110	158	146	
35	7	82	147	127	162	159	157
37	7	81	166	118	166	157	153
	Av.	76	157	113	164	141	135

[1] Two intraperitoneal injections of glucose (200 gm. per 100 gm. of body weight) were given, the second injection being given immediately after the 90-minute blood sample was taken.

injection is of great interest. Cole and Harned (19) have reported that in a Yale strain of rats the glucose tolerance curve changes markedly after 50 days of age, after which period diminished glucose tolerance was demonstrated. In the technic used by these authors 350 mg. of glucose per 100 gm. of body weight were injected. In the present studies no changes in the glucose tolerance correlated with age were found in the untreated rats, when only 200 mg. of glucose per 100 gm. of body weight were injected. However, when a glucose tolerance test in which 2 injections of glucose, each consisting of 200 mg. per 100 gm. of body weight were given, a difference in the response between the untreated young and the adult rats appeared. This is shown in table 8, in which it is apparent that following the second injection of glucose, the blood sugar falls more rapidly in the young rats than in the adults. These differences in findings between the response of the very young and the adult rat may be related to differences in insulin production. Waters and Best (20) report decreasing insulin content of the pancreas with age in the cow.

SUMMARY

Glucose tolerance tests were performed on rats which were injected with a saline extract of the anterior pituitary. The changes in the glucose tolerance curves which were found differed with the age of the animal and the duration of treatment.

In the adult rat a diabetic type of glucose tolerance curve was found 24 hours following a single injection of anterior pituitary extract. With continued administra-

tion of the extract the glucose tolerance test showed a more rapid fall of the blood glucose concentration than in control tests.

Thirty-day-old rats were also injected with anterior pituitary extract. In these animals a diabetic type of glucose tolerance curve was never observed. Following several daily injections of A.P.E., an increased tolerance to glucose was found which persisted with continued treatment. When the A.P.E. was discontinued a gradual return to the normal type of glucose tolerance curve occurred.

The significance of these results with respect to the resistance of the rat to the diabetogenic effects of extracts of the anterior pituitary has been discussed.

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THE PREPARATION AND PROPERTIES OF UROGASTRONE[1]

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UROGASTRONE is a water-soluble substance, obtainable from the urine of both dog and man, which effectively inhibits gastric secretion (1, 2, 3). Its physiological rôle has not been elucidated as yet, but in the course of preparing and assaying several hundred preparations of urogastrone, some information regarding its chemical and physiological properties has been obtained. It is the purpose of this report to communicate these findings and to describe methods for obtaining the principle in highly potent form.

METHODS OF PREPARATION

Crude concentrate. The urine is collected in 5-gallon bottles containing sufficient preservative to maintain sterility when the bottle has become filled. In the case of tricresol a concentration of 0.25% is used; with phenol, a concentration of 0.5%, with toluene, enough is used to cover the surface. If the latter preservative has been used, the urine without stirring is siphoned off to separate it from the toluene, which would otherwise interfere with the process of extraction. The urine is made faintly acid to Congo Red paper with glacial acetic acid. With continuous stirring, 50 cc. of a saturated acetone solution of benzoic acid are added per liter of urine. After an additional 10 minutes of stirring the mixture is allowed to stand overnight in the cold. The next morning the clear supernatant fluid is siphoned off and discarded and the precipitate is collected on large suction filters. The precipitate is re-suspended in a quantity of saturated aqueous solution of benzoic acid equal to 1/10 of the original volume of urine. The mixture is refiltered by suction and allowed to remain on the filter for several hours in order to remove as much water as possible. If necessary, the material in this form may be stored in the cold for several days. The precipitate is then mixed with acetone in an amount equal to 1/20 of the original volume of urine and 0.5 cc. of 5% NaCl is added per liter of urine. On either a volume or weight basis the added acetone should contribute at least 80% to the final mixture; if this is not the case, more acetone should be added. After standing for several hours the clear brown supernatant fluid is siphoned off and discarded and the precipitate is collected by centrifuging. The precipitate is washed thoroughly with acetone and ether and dried. In order to aid the next extraction step, it is desirable to pulverize finely the resulting product.

This procedure has been used routinely in handling 100 liters or more of urine per day for over a year and has been found to be both reliable and convenient. The yield is such that 1 gm. of extract represents from 12 to 15 liters of urine. It is a gray-tan

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powder, not soluble in water unless distinctly alkaline. It appears to consist mainly of a mucoid type of protein, since it yields reducing substances on hydrolysis, is insoluble in 70% alcohol, but is not precipitated by trichloracetic acid. Since neither this product nor the original urine is suitable for assay, the recovery of activity cannot be directly determined. However, there is reason to believe that the yield is not quantitative.

Initial purification. A method for purifying the crude concentrate, has been published (1). It consists essentially of dissolving the active fraction in 70% acidified acetone and precipitating it by raising the acetone concentration to 90%. This procedure was found to be less than quantitative, and inconvenient to use on a large scale because of the gummy nature of the final precipitate. The following modification was devised to overcome these objections.

One gm. of crude concentrate is thoroughly shaken with 15 cc. of 70% acetone containing 1 cc. of concentrated HCl per 100 cc. The mixture is centrifuged and the residue is re-extracted with another 15 cc. portion of the acetone mixture. From the combined supernatant extracts the active material is precipitated by the addition of 1 volume of ethyl alcohol, containing 3 cc. of aniline, and 3 volumes of acetone. The flocculent precipitate is collected by centrifuging and washed with acetone and ether and dried.

The final product is a tan powder not very soluble in water because it is at its isoelectric point. The addition of acid or alkali makes it very soluble. The yield by weight from crude concentrate is about 10%. Since the crude concentrate is not suitable for assay, there is no reliable way of determining the yield of activity. The product is active in 5 mg. doses, representing 600 to 800 cc. of urine.

Further purification. A considerable number of procedures of various types have been used to accomplish a further purification. The most satisfactory method from the standpoint of yield, purification, and consistent behavior is as follows. One gm. of the initially purified product is dissolved in 10 cc. of distilled water with the aid of 0.2 cc. of concentrated HCl. The addition of 20 cc. of acetone aids the process of solution. The further slow addition of 20 cc. of acetone, which raises the acetone concentration to 80% brings down a gummy precipitate. The addition of 0.5 cc. of pyridine increases the precipitate and converts it to a tarry material. The mixture is centrifuged and the light-colored supernatant fluid is separated. The residue is subjected to the same extraction procedure 3 more times. The combined supernatant extracts are then distilled to dryness in vacuo at a temperature below 55° C. The dried residue is taken up in the least quantity of methyl alcohol acidified with 1 cc. of concentrated HCl per 100 cc. The methyl alcohol solution is centrifuged to remove a small amount of insoluble material, which in turn is washed with the least quantity of acid methyl alcohol. The original methyl alcohol solution and the washings are combined. The active material is precipitated from this solution by the addition of 1 volume of normal butyl alcohol, containing 2 cc. of aniline, and 3 volumes of acetone. The flocculent precipitate is first washed with acidified acetone to convert it into the chloride, then with ether, and dried.

The final product is a tan powder, very soluble in water. The yield by weight is in the neighborhood of 10%; the yield of activity is nearly quantitative. The product is active in doses of from 0.25 to 0.50 mg.

Some of the above material has been purified further by subjecting it to a procedure similar to the above, except that aniline was substituted for the pyridine. This product assayed 1 'dose' per 0.2 mg. and is the most potent preparation which has so far been obtained.

Chemical Properties

Urogastrone is completely insoluble in organic solvents which are immiscible with water, such as ether, chloroform, benzene, and petroleum ether. It is insoluble in absolute acetone, pyridine, and glacial acetic acid, but is quite soluble in methyl alcohol and ethylene glycol, and slightly soluble in butyl alcohol. It is soluble in dilute acetone and pyridine up to at least 85%; it is soluble in slightly higher concentrations of ethyl alcohol. It is more soluble in aqueous acetone or alcohol when acidified and is precipitated from such solutions by aniline, but not by pyridine or NaOH.

Urogastrone is precipitated from aqueous solution quantitatively by phosphomolybdic acid and nearly so by picric and tannic acids. It is only partially precipitated by trichloracetic acid, saturation with ammonium sulfate, or isoelectric precipitation, and scarcely at all by sulfosalicylic acid. Urogastrone is adsorbed in the crude state by benzoic acid and zinc hydroxide, but when purified, these reagents are no longer effective.

None of the above-mentioned reagents destroys urogastrone. It is stable to boiling for 5 minutes, although prolonged boiling probably destroys it. It withstands acids readily, but alkali less readily. It is not inactivated by incubation with pepsin in N/10 HCl.

The behavior of urogastrone with respect to precipitants and solvents, and its stability to boiling and peptic digestion suggests that the active agent is not a protein. It appears to be some kind of organic base of considerably less complexity than peptone.

Physiological Properties

Urogastrone is routinely assayed in dogs with pouches (vagotomized) of the entire stomach, although the Heidenhain type of pouch has also been used. For this assay the dogs are placed in stocks in the forenoon and observed until they no longer secrete free acid. They are then given subcutaneously a dose of histamine, which previous experience has indicated will cause the secretion of acid equivalent to approximately 30 to 40 cc. of N/10 HCl in 1 hour. An interval of 2 hours is allowed, during which the secretion of free acid uniformly stops. The urogastrone preparation is then injected intravenously, followed 10 minutes later by the original dose of histamine subcutaneously. The quantity of free acid secreted during the following hour is determined and compared with the response to the histamine in the forenoon. Inhibition is expressed as the percentage reduction in acid output. Usually at least 6 dogs are used for an assay and the results are averaged. Inhibition of from 50 to 75% is considered the most desirable range. The potency of the preparation is determined from a previously established curve, which relates the percentage inhibition to the number of 'doses.' A 'dose' is defined as the quantity which produces 50% inhibition under the conditions of the assay. The term 'dose' is preferred to 'unit' because the latter carries the implication of absolute accuracy.

Under these conditions urogastrone is very effective in temporarily inhibiting the output of acid by the gastric glands. Since histamine is the most potent stimulus for gastric secretion, urogastrone is probably more effective against other types of stimuli. It has been found to be active when given subcutaneously, provided 5 to 10 times the effective intravenous dose is administered 30 minutes before the histamine. Urogastrone has been given by tube directly into the duodenum of total pouch dogs in doses of 50 secretory 'doses' without yielding any inhibition of gastric secretion. Urogastrone has been shown to be effective in inhibiting gastric secretion in man when given subcutaneously (4).

Urogastrone is apparently unable to inhibit gastric secretion in the frog, a species which lacks true parietal cells. This was demonstrated in two ways. Gastric contents were obtained from intact frogs by means of a small rubber tube attached to a medicine dropper. When the fasting contents on successive sampling proved to be neutral, 0.5 cc. of distilled water (histamine was unnecessary) was injected into the ventral lymph sac. In each of 24 frogs the gastric contents contained free acid within 30 minutes. In 6 other frogs the appearance of free acid was neither delayed nor prevented by the inclusion of a third of a 'dose' of urogastrone in the distilled water. In confirmation of these results urogastrone has been found to be incapable of inhibiting the in vitro secretion of acid by the isolated gastric mucosa of the frog, employing the technique recently described (5).

TABLE 1. RELATION BETWEEN SECRETORY AND MOTOR POTENCY OF UROGASTRONE

Preparation	Mg. per 'Dose'	Motility Assay				Min. of Inhibition per 'Dose'
		No. assays	Dose injected		Min. of inhibition	
			mg.	'Doses'		
1	3.7	5	5	1.35	1.2	0.9
2	2.1	15	3	1.43	6.4	4.5
3	1.0	8	1	1.00	3.8	3.8
4	0.27	6	1	3.70	8.3	2.2

The inhibitory effects of urogastrone preparations on gastric motility stimulated and maintained by distention of the recording balloon with approximately 80 cc. of air have been investigated in dogs. This type of motility is preferred to hunger contractions because it is more easily elicited, more predictable in its behavior, and more analogous to digestive motility (6). The degree and duration of motor inhibition obtained with urogastrone preparations are not striking. Doses which inhibit gastric secretion for an hour or more reduce motility for only a matter of minutes; with many of the less refined preparations the degree of inhibition is so slight or so variable in duration as to be questionable. For this reason it is difficult to compare the motor and secretory potencies of preparations of various types and at various stages of purity. The data in table 1 suggest the possibility that 2 separate factors are involved. This possibility is somewhat strengthened by the observation that motor inhibition, in contrast to secretory inhibition, is not demonstrable in the vagotomized stomach.

Urogastrone has been studied from the standpoint of possible toxic actions. The assay animals have been injected with various preparations several times a week over periods of a year or more, without evidence of cumulative toxicity. The response of the assay dogs to the control injections of histamine have been carefully followed over periods of months and years (7). Since there has been no tendency for the response to change, urogastrone exerts neither cumulative nor toxic effects on gastric secretion.

The early preparations of urogastrone contained a pyrogenic substance, which produced a febrile reaction in the assay animals. The pyrogenic factor was found to be mainly of bacterial origin (1). Although the methods of preparation described here yield pyrogen-free urogastrone, it is possible that traces of bacterial products remain. The subcutaneous injection of urogastrone preparations to human subjects produced a delayed local reaction consisting of a simple erythema (4). Similar reactions can be obtained with small doses of bacterial vaccines and bacterial culture filtrates (8). The severity of the local reaction has diminished as the preparations have been further purified.

Doses of urogastrone which inhibit gastric secretion are without effect on blood pressure; multiples of this dose, however, are apt to produce a characteristic slight fall in blood pressure, which is very gradual in onset and recovery. The agent responsible for this depressor effect has been found to be non-dialyzable, stable to boiling for 5 minutes at neutral reaction or in N/10 HCl, but destroyed by boiling in N/10 NaOH. In these respects it resembles depressan, a substance present in normal urine, discovered and characterized by Wollheim (9). The depressor substance in urogastrone preparations is precipitated by trichloracetic and picric acids, in contrast to depressan, but this difference is probably more apparent than real, since the stage of purity greatly influences these reactions. The depressor substance is not histamine, since it does not give the proper type of blood pressure response; it is not adsorbed by permutit, or destroyed by histaminase. However, minute traces of histamine may

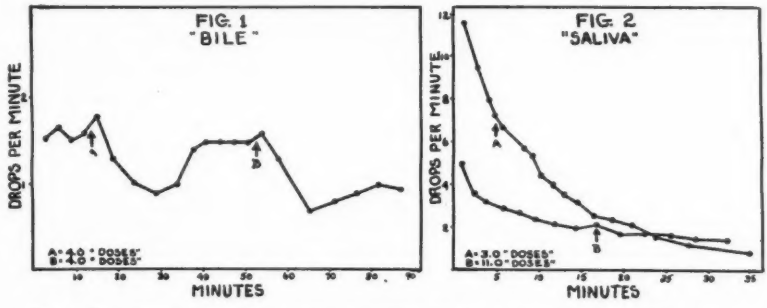

Fig. 1. Effect of urogastrone on spontaneous flow of bile in acute bile-fistula dogs under nembutal anesthesia. At *A* and *B*, 4.0 'doses' of urogastrone were injected intravenously.

Fig. 2. Effect of urogastrone on pilocarpine-induced salivary secretion in acute salivary-fistula dogs under nembutal anesthesia. At *A* and *B*, 3.0 and 11.0 'doses' of urogastrone respectively, were injected intravenously.

be present, since Code extracts of crude urogastrone contract the guinea pig's ileum, and more purified preparations give a positive trypan blue test (10) when used in high concentrations.[2]

The question of whether the action of urogastrone is specific for the stomach or whether it acts on other digestive secretions has also been investigated. The salivary ducts were cannulated in dogs under nembutal anesthesia and pilocarpine was injected intravenously in order to provoke a copious flow of saliva. During this response urogastrone was administered intravenously in quantities of from 2 to 11 'doses.' In 8 trials the course of the salivary response was apparently unchanged (fig. 2). Urogastrone, therefore, does not produce an abrupt or a complete inhibition of salivary secretion, although a gradually developing partial inhibition would not have been detected by the procedures used.

In similar acute experiments urogastrone in quantities of from 2 to 4 'doses' was found to inhibit the spontaneous flow of bile from the liver. The degree of inhibition varied, but occurred in each of 12 trials. The most striking response is illustrated in fig. 1. Similar results were obtained in the case of pancreatic secretion, except that the effect was demonstrated as a diminution in the response to successive injections of a standard dose of secretin. Since in most of these experiments a slight fall in blood pressure occurred, it is not certain that urogastrone itself is responsible for the effect

[2] We are indebted to Doctors M. Rocha e Silva and F. R. Götzl for these determinations.

on secretion. Solution of this problem must await the study of more highly purified material.

On the basis of its physiological and chemical properties urogastrone has been differentiated from a number of biologically active substances which may be present in urine. Urogastrone failed to produce any evidences of gonadotropic or estrogenic activity when administered subcutaneously to 10 21-day-old rats in 3 daily injections of 3 'doses' each. Urogastrone has been differentiated from posterior pituitary principles by a comparison of their effects on gastric secretion, gastric motility, blood pressure, and urine excretion (11). Since urogastrone is comparatively stable to heat and acids and does not produce a rapid fall in blood pressure, it is distinguishable from kallikrein (12). It cannot definitely be said at present whether urogastrone is related to the substance discovered by Sandweiss et al. (13), which prevents or delays the development of ulcers in Mann-Williamson dogs. The effective dose for the prevention of ulcer is much smaller than the dose required to inhibit secretion. Extracts of urine from ulcer patients, although ineffective in preventing ulcers, do apparently inhibit gastric secretion (2). In our experience, the output of urogastrone is much reduced in patients with peptic ulcer (14).

SUMMARY

Methods are described for the preparation of extracts containing urogastrone from normal urine. From its chemical behavior the active agent appears to be neither a protein nor a polypeptide susceptible to the action of pepsin, but is probably a complex organic base. On intravenous injection into dogs with pouches (vagotomized) of the entire stomach, total doses of less than 0.5 mg. were capable of inhibiting the gastric secretory response to histamine. The extracts produced a mild inhibition of gastric motility, but only if the vagi were intact. It is possible that separate factors are responsible for the motor and secretory effects. The best available preparations inhibited pancreatic and biliary secretions, but not salivary secretion. The active agent has been differentiated from a number of substances found in urine, including estrogenic and gonadotropic hormones, the principles of the posterior lobe of the pituitary, and the vasodilator, kallikrein. The preparations contained traces of a depressor substance and a factor which produced a delayed local erythema on subcutaneous injection in man.

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THE PRIMARY ACTION OF THE PARATHYROID HORMONE[1]

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WHETHER THE PARATHYROID HORMONE acts primarily on calcium or on some phase of phosphorus metabolism has been the subject of a great deal of discussion. Since there is a close interrelationship in the metabolism of these inorganic elements, the question is difficult to settle. There has been an attempt to associate parathyroid action with the excretion of phosphate by the kidney. Tweedy and his associates find no evidence that parathyroid extract produces any action on blood and tissue calcium in nephrectomized and ureter-ligated dogs (1, 2). Observations contrary to this have been made (3, 4). Some are based on histological changes of bone, but these may be of doubtful significance. We deemed it advisable to carry out a series of experiments on normal, nephrectomized and ureter-ligated animals following parathyroid extract treatment, with a view of obtaining further information on this controversial problem. The results to be reported support the view expressed by Albright and his associates (5, 8) that primarily the hormone acts on phosphorus metabolism.

METHODS

Rats, cats and dogs were used in these studies. The rats were adult males of a hooded strain. Nephrectomy and ligation of renal vessels or of the ureters were performed after a single midline incision, ether anesthesia being used in rats and nembutal in cats and dogs. All the animals were fasted from 16 to 24 hours prior to the test or the operative procedures until the termination of the experiment. Water was given ad libitum throughout the experimental periods.

Serum calcium determinations were carried out by the Clark and Collip method (9), serum inorganic phosphate by the Fiske and Subbarow method (10) adapted to the Evelyn photocolorimeter. Blood haemoglobin readings were made with the photocolorimeter. These have been omitted from the data since variations observed (with the exception of one cat) were small. Most of the analytical procedures were made in duplicate and only the average results are given.

The parathyroid extracts used (*extract* 583, 587 and 591) were prepared from acetone-dried cattle parathyroid glands, or from fresh frozen glands, by standard hydrolytic procedure. Unless otherwise stated, all the injections of parathyroid extract were made subcutaneously. The cats and dogs used served as their own controls, as assays were carried out on them from 2 to 4 weeks before the final experiment. The posterior pituitary extract used was a hot 0.25% acetic acid extract of acetone-dried posterior pituitary gland tissue.

RESULTS

Initially a number of experiments were carried out on groups of normal, nephrectomized and ureter-ligated rats (table 1). The dose and the irregularity of response in

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the normal animals are in fair agreement with the observations of Pugsley (11). The results show that following nephrectomy or ligation of the ureters no rise in serum calcium was observed as a result of parathyroid hormone injections. Serum inorganic phosphate showed a marked rise throughout the postoperative period. It may be pointed out that in our experience nephectomized fasted adult rats survived from 3 to 4 days with an approximate 50 to 60% mortality on the 3rd day.

The effect of parathyroid extract on the serum calcium and inorganic phosphate of dogs following nephrectomy and ligation of the renal vessels or of the ureters is shown in figure 1. Even repeated injections of parathyroid extract did not elicit a significant rise in serum calcium in animals deprived of their kidney excretory mechanism. The slight changes observed can probably be ascribed to variations in hemoconcentration. The rise in serum inorganic phosphate following nephrectomy or ureter ligation was less than that observed in rats. This may be associated with the rate of

TABLE 1. PARATHYROID HORMONE EFFECT IN NORMAL, NEPHRECTOMIZED AND URETER-LIGATED RATS

Parathyroid 583 daily, cc.	Hr. following 1st injection	No. of animals	Serum calcium, mg./100 cc.		Serum phosphate mg./100 cc.	
			Range	Av.	Range	Av.
			Normal			
—	—	12	9.7–11.0	10.3	2.6– 6.1	5.2
5	16	4	9.8–10.7	10.5	2.0– 5.9	4.8
5	24	4	10.6–12.3	11.9	1.8– 5.7	3.5
5	48	8	11.3–15.3	14.4	2.0– 5.3	3.7
5	72	6	11.6–14.1	13.6	2.6– 6.1	3.6
			Nephrectomized, 1 hr.			
5	16	8	9.7–10.4	10.1	7.3–14.5	11.0
5	24	10	9.2–10.7	9.7	11.0–27.3	14.1
5	36	6	9.3–10.5	9.8	11.1–19.2	15.8
5	48	8	9.3–10.2	9.6	13.0–22.4	18.3
5	72	5	8.7–10.1	9.5	21.2–28.4	23.3
			Ureters ligated, 1 hr.			
5	24	4	9.6–10.2	10.0	13.1–15.5	13.6
5	48	4	9.6–10.1	9.7	16.4–20.5	17.7

turnover in these 2 species. The rate of disappearance from the blood of intravenously administered calcium in the form of calcium gluconate appeared quite normal. This may not be of marked physiological significance in view of the time that had elapsed since the loss of kidney function. *Dogs* 5 and 6 were allowed to drink 150 cc. of 0.9 NaCl at the 14, 22 and the 48-hour periods without any noticeable effect.

The results obtained with 2 cats (fig. 2), one nephrectomized and the other ureter-ligated, are essentially the same as those observed with the dogs and rats. In *cat* 2 both ligated ureters were brought outside the abdominal wall. On cutting them 22 hours following the first injection of parathyroid extract a flow of urine was observed. Subsequent to a further injection of extract this animal showed a normal rise of serum calcium. This latter is in agreement with an observation on one dog by Tweedy and his associates (2). For some unknown reason *cat* 1 showed a marked haemodilution associated with a constant decrease of serum calcium. *Cat* 2 died on the 5th day following the operation, apparently from kidney dysfunction due to infection. Microscopically the kidneys showed advanced degeneration and haemorrhage into the tubules and glomeruli.

Unfortunately, no autopsies were carried out on *dogs* 2 and 3. *Dogs* 5 and 6 showed at autopsy a marked haemorrhagic gastric mucosa. On the other hand, *cat* 1 showed no such changes. These observations are contrary to those of Tweedy, et al.

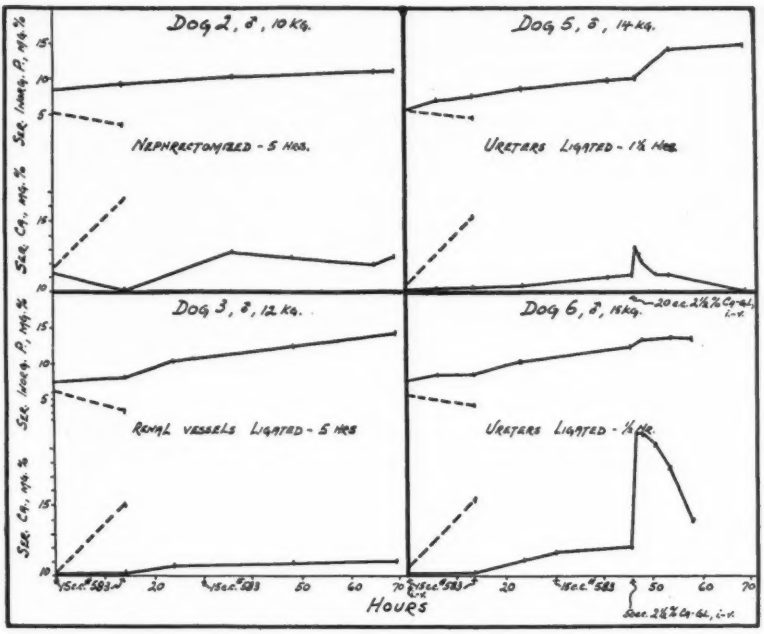

Fig. 1. Parathyroid hormone action in nephrectomized and renal vessels or ureter-ligated dogs. Dotted lines represent the control, solid lines the experimental tests.

(1, 2) and the suggestion that this overdosage effect may be a result of the simultaneous presence of hypercalcaemia and hyperphosphataemia may have to be modified (12, 13).

The results reported, together with those of Tweedy and his associates (1, 2), definitely associate the rise of serum calcium following parathyroid extract with normal kidney function. Further, they rather support the view that the parathyroid

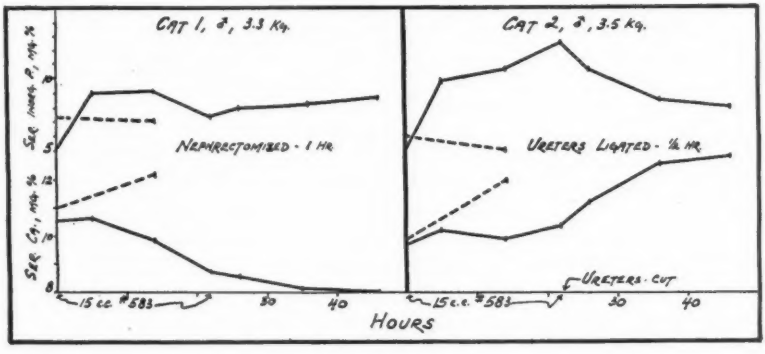

Fig. 2. Parathyroid hormone action in nephrectomized and ureter-ligated cats. Dotted lines represent control, solid lines the experimental tests.

hormone primarily affects phosphorus metabolism. With this in view the experiments reported in fig. 3 were carried out by administering to normal dogs, following treatment with parathyroid extract, sodium acid phosphate intravenously at such a rate as to maintain a constant serum phosphate level. It will be observed that in preventing a fall of serum phosphate the customary rise of calcium did not occur. Blood PH determinations were not carried out so it is not known to what extent this may have affected the results.

Since hypercalcaemia due to parathyroid hormone injection appears to be dependent on an active kidney secretory mechanism (probably phosphate excretion), it

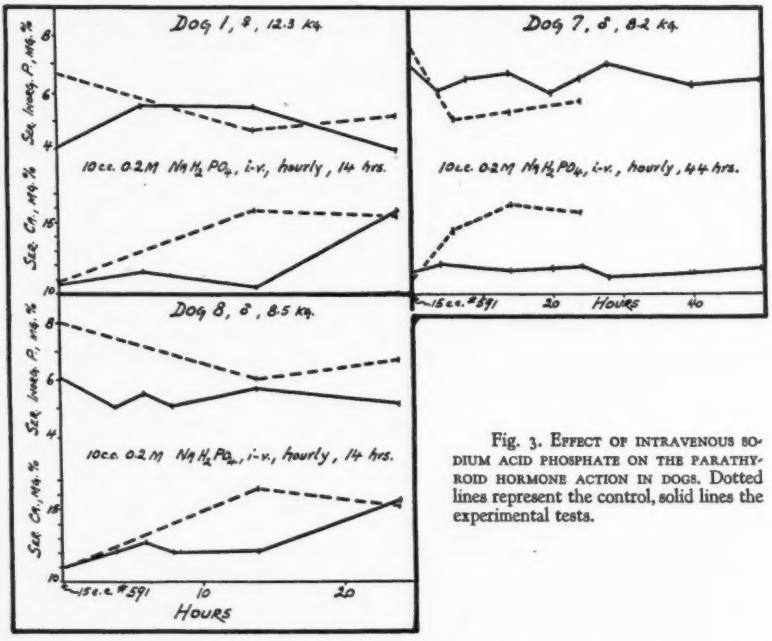
Dog 1, ♀, 12.3 kg.
Dog 7, ♂, 8.2 kg.
Dog 8, ♂, 8.5 kg.
Ser. Inorg. P, mg. %
Ser. Ca, mg. %
10 cc. 0.2 M NaH_2PO_4, i.v., hourly, 14 hrs.
10 cc. 0.2 M NaH_2PO_4, i.v., hourly, 44 hrs.
15 c.c. #591
Hours

Fig. 3. EFFECT OF INTRAVENOUS SODIUM ACID PHOSPHATE ON THE PARATHYROID HORMONE ACTION IN DOGS. Dotted lines represent the control, solid lines the experimental tests.

was thought that an antagonism might exist between the posterior pituitary antidiuretic substance and the parathyroid hormone. The results obtained on 4 dogs treated with both parathyroid extract and posterior pituitary extract are shown in figure 4. Serum phosphate was maintained in these dogs at a nearly constant level, while the serum calcium showed no appreciable change throughout the period of anuria. In dog 4, however, complete anuria was not established, since during the first 8 hours of the experiment the animal passed 200 cc. of urine. In this animal an approximate 50% of the normal hypercalcaemic response was elicited. Evidently there is nothing to suggest a direct antagonism of posterior pituitary gland extract to the parathyroid hormone.

DISCUSSION

There have been two schools of thought as to the mechanism whereby parathyroid extracts affect calcium and phosphorus metabolism. One holds that parathyroid primarily affects calcium metabolism by acting on the osteoclasts of bone

tissue (14, 15). The other holds that the primary effect is on phosphorus metabolism (5–8). No attempt will be made to give a complete discussion of these 2 theories since a number of excellent reviews are available (8, 13, 16, 17).

It has been established by Tweedy and his associates and in the present communication that parathyroid extract has little or no effect on serum calcium in nephrectomized animals. Similar results were obtained after ligation of the renal vessels or of the ureters. The reestablishment of urine flow by cutting the ligated ureters of the experimental animal injected with parathyroid extract resulted in a subsequent rise of

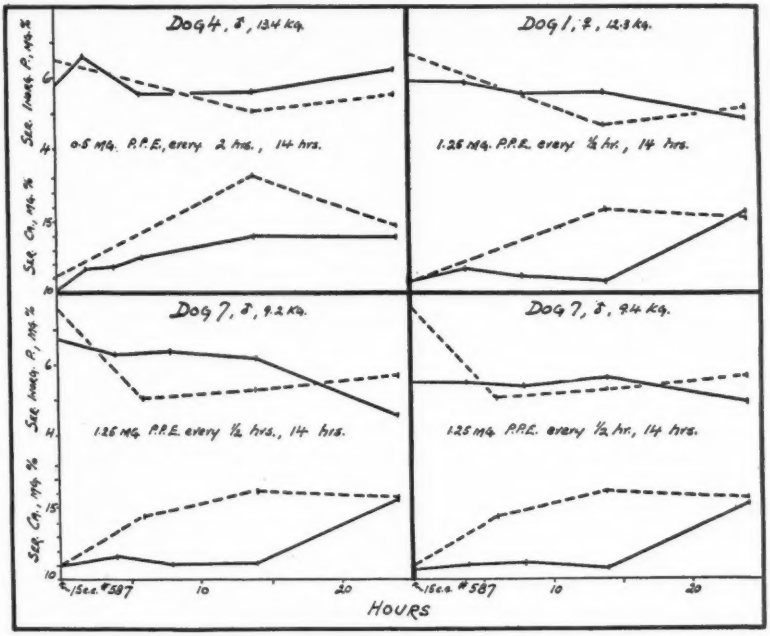

Fig. 4. Effect of posterior pituitary extract on the parathyroid hormone action in dogs. Dotted lines represent the control, solid lines the experimental tests.

serum calcium. Finally, during complete anuria in dogs as a result of treatment with posterior pituitary exact, no rise in serum calcium was observed following parathyroid hormone treatment. These observations associate the hypercalcaemia of parathyroid extract with the excretory function of the kidneys. We have made repeated attempts to obtain an extract from renal tissue which by itself or together with parathyroid preparations would affect the serum calcium of nephrectomized animals. All such attempts have given negative results.

Clinical as well as experimental evidence has been brought forward in favor of the view that all, or at least part, of parathyroid hormone action is a direct effect upon the excretion of phosphate by the kidney (5–8, 18–20). This is based chiefly on the observations that the administration of parathyroid extract resulted in an immediate increase in the excretion of phosphates, followed successively by hypophosphataemia, hypercalcaemia and hypercalcuria. On discontinuing substitution therapy with parathyroid extract these effects were altered in the opposite direction. It has been estab-

lished, however, that the rise in serum calcium as a result of hormone treatment is not invariably accompanied or preceded by a decrease in serum phosphate (21). Nevertheless an increased excretion of phosphate may not necessarily be associated with a decrease in serum phosphate, especially since the latter represents the equilibrium between the rates of absorption and excretion. Our observations support the above-mentioned view. Further support is the observation that the administration of parathyroid hormone to dogs followed by repeated intravenous injections of sodium acid phosphate to prevent an actual decrease of serum phosphate resulted in no change of serum calcium. The observation by Brull and Carbonesco (22) that the kidney from a parathyroidectomized dog excretes less phosphate than does one transplanted from a normal dog and perfused with the same blood at the same time further indicates a direct action of the parathyroid hormone upon the excretion of phosphate by the kidney.

Attempts have been made to show that the action of the parathyroid hormone on kidney (probably an increased phosphate diuresis) may be distinct and independent of an action in mobilizing calcium from the skeleton (18, 23). The evidence presented does not suggest any direct effect on calcium mobilization from bone. Judging from histological studies by a number of authors there may be good reason to question the merit of the hypothesis that the hormone acts on bone tissue, notably the osteoclasts, (24–28). Further light may be thrown on this subject by an extension of studies relating to the effect of the parathyroid hormone on the enzyme systems concerned with the metabolism of phosphorus, the phosphatases (13, 29, 30).

SUMMARY

Parathyroid extracts produced no effect on the serum calcium in rats, cats or dogs immediately following nephrectomy or ligation of the renal vessels or of the ureters Reestablishment of urine flow in one cat by cutting the ligated ureters was followed by a normal parathyroid hypercalcaemia. During anuria induced by posterior pituitary extract no parathyroid hypercalcaemia was noted. Injection of parathyroid extract followed by repeated intravenous administrations of sodium acid phosphate to maintain a constant serum phosphate level prevented the customary rise of serum calcium. The evidence given does not suggest a direct effect of the parathyroid hormone on bone. These results rather favor the theory that the primary action of the parathyroid hormone is upon the excretion of phosphate by the kidneys.

We wish to thank Dr. R. L. Noble for assistance with the surgical procedure and to acknowledge the technical assistance of Mr. E. A. Andersen.

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CAPACITY OF RAT'S LIVER TO INACTIVATE DESOXYCORTICOSTERONE ACETATE

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CERTAIN CRYSTALLINE ESTROGENS AND ANDROGENS (1–7) and the endogenous ovarian estrogens and testicular androgens of the rat (8–12) are inactivated by the liver. Such evidence is used to explain the fact that natural estrogens and androgens are relatively ineffective when administered by mouth.

Kuizenga, Nelson and Cartland (13) found that 2 of the adrenal steroids, corticosterone and dehydrocorticosterone, as well as certain adrenal cortical extracts were just as effective orally as when given subcutaneously. This indicates that these adrenal substances, unlike the gonadal hormones, are not inactivated by the liver. Desoxycorticosterone acetate, on the contrary, was found to be much less effective orally than parenterally. By transplantation experiments in the rat, Eversole, Edelmann and Gaunt (14) have shown that the endogenous adrenal hormone or hormones necessary for maintenance of life are not inactivated by the liver. These results are therefore in agreement with the demonstrated undiminished potency of the adrenal cortical extracts when given orally. Eversole and Gaunt (15) tested the effect of liver on desoxycorticosterone acetate by implanting pellets of this substance (with cholesterol) into the spleen of adrenalectomized rats. They concluded that it was not inactivated by the liver of the rat. The present report concerns experiments of a similar nature.

PROCEDURE

Male rats weighing 100 ± 10 gm. were bilaterally adrenalectomized by the lumbar route and were divided into 4 groups. One group served as untreated controls, the other groups received pellets of desoxycorticosterone acetate which were located subcutaneously in one group and intramesenterically in the others.

The desoxycorticosterone pellets were cylindrical in shape. They were prepared by compression of pure crystalline desoxycorticosterone acetate without a binder.[1]

In the subcutaneous implant group the pellets were inserted through the adrenalectomy incision into a subcutaneous pocket made by blunt dissection in the dorsal neck region.

In the mesenteric implant groups a loop of small bowel was delivered through an abdominal incision and spread out on a piece of cotton soaked in normal saline. The pellet was placed in the center of the mesentery, which was then gently folded so that the pellet lay between two layers of mesentery. A slip knot of silk thread was then placed over the covered pellet and tightened only enough to hold the pellet in place without occluding blood vessels. This operation was quickly and easily performed and proved to be efficient since at autopsy the pellets were found in the same position as when implanted, surrounded by healthy mesentery. Adhesions to other struc-

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[1] The pellets were prepared and furnished by the Schering Corporation, Bloomfield, N. J.

tures were never encountered, therefore the venous drainage from the pellet area was exclusively portal and thus directly into the liver.

The control groups were subjected to mock abdominal operations involving comparable manipulation of the bowel. All animals were given the regular stock diet of Rockland rat pellets and water ad libitum. Daily weights were recorded and the last weight of the living animal was used in computing the weight changes.

The survival period was determined to the nearest half day. Operations were performed in the afternoon, therefore an animal was considered to have survived one whole day if still alive the next afternoon; if found dead the following morning, its survival was considered to be 1.5 days. The data from animals which survived less than 24 hours were excluded since death at this early period was probably due to shock. The experiment was arbitrarily terminated at 30 days and the surviving animals were killed. All animals were autopsied and a thorough search for adrenal tissue was made. The data from those animals having accessory or regenerated adrenal tissue were excluded. The pellets were recovered and dried in an oven before weighing.

RESULTS

The results are summarized in table 1. Eight of the 12 animals with subcutaneous pellets (66%) were in excellent condition when killed 30 days after the operation and

TABLE 1. SURVIVAL OF ADRENALECTOMIZED RATS FOLLOWING SUBCUTANEOUS AND INTRAMESENTERIC IMPLANTS OF DESOXYCORTICOSTERONE ACETATE

Group No.	Group treatment	No.[1] animals	Animals surviving 30-day exper. period	Av. survival period		Av. total wt. change	Av. daily absorption from pellet
			%	*days*	*range*	*gm.*	*mg.*
1	Untreated adrenalectomized controls	12	0	5.08	2.5– 7.5	− 7.9	—
2	Subcutaneous pellets (av. wt. 23.7 mg.)	12	66	22.29[2]	4.5–30.0	+65.8	0.188
3	Intramesen. pellets (av. wt. 22.6 mg.)	9	0	5.56	1.5–11.5	−10.6	0.170
4	Intramesen. pellets (av. wt. 80.9 mg.)	18	33	20.19[2]	1.5–30.0	+17.8	0.559

[1] Not including animals in which regenerated or extraneous adrenal tissue was found at autopsy.
[2] Including those animals which were killed after 30-days' survival.

showed an average weight gain of 99.5 gm. for the experimental period. The average weight change of the entire group, including the 4 which died (average survival 6.9 days) was a gain of 65.8 gm.

It is evident that the subcutaneous pellets were adequate to maintain life with weight gain in the majority of animals. Pellets of the same size, when implanted intramesenterically, however, displayed no such ability. All 9 animals in this group (*group* 3) died during the experimental period. The longest survival period was 11.5 days (one animal) and the average for the group was 5.56 days, which is practically identical with that of the untreated adrenalectomized controls (*group* 1), which was 5.08 days. The average body weight change of this intramesenteric group was a loss of 10.6 gm. which corresponds to the average loss of 7.9 gm. in the untreated control group.

The difference between this intramesenteric group and the subcutaneous group cannot be due to different rates of absorption from the pellets in the two locations since the average daily absorption was approximately the same in the two groups (table 1). The difference between the two groups in the effectiveness of the desoxy-

corticosterone acetate must therefore be due to the different routes of absorption of the hormone. The quantity of desoxycorticosterone acetate which maintained life with weight gain in the majority of animals when absorbed into the peripheral blood stream did not do so when absorbed directly into the portal blood stream. This result implies that the liver inactivated the desoxycorticosterone acetate from the intramesenterically located pellet. In view of the fact that desoxycorticosterone acetate has some oral effectiveness (less than 1/35th of its parenteral potency) it was thought possible that the capacity of the liver to inactivate this substance may be limited, so that amounts which exceed the limit pass through without inactivation. It was decided to test this hypothesis by using larger amounts of the hormone.

Accordingly, another group (4) of 18 adrenalectomized males were given intramesenterically located pellets which averaged approximately 80 mg. Since a single pellet of such size would have been unwieldy, pellets of approximately 20 to 50 mg. were combined so that 2, 3 or 4 pellets equalled the required amount. The procedure employed in this group was identical with that already described. No difficulty was experienced in implanting the several pellets which were stationed at intervals in the mesentery of the small bowel.

As would be expected, there was a slight difference in the daily amount of hormone absorbed from the pellets, depending on the number of pellets implanted; 2 pellets yielded an average of 0.476 mg. daily, 3 pellets 0.636 mg. and 4 pellets 0.690 mg. However, since no marked differences between these three sub-groups were observed in the results, they are considered together in the table.

Of the 18 animals receiving the larger amounts of hormone, 6 were in excellent condition when killed 30 days after the beginning of the experiment. This survival of 33% was not as good as the 66% survival obtained when the smaller amount of hormone was located subcutaneously, but was better than the absolute lack of survival in the untreated control group and the small pellet intramesenteric group. In other respects, also, this group was better than the untreated control group and was intermediate between the subcutaneous group and the small pellet intramesenteric group. The average weight gain of the 6 survivors in this group was 50 gm., whereas in the subcutaneous group it was 99.5 gm. The average weight gain for this whole group was 17.5 gm. in contrast to an average gain of 65.8 gm. for the subcutaneous group.

DISCUSSION

These data demonstrate that desoxycorticosterone acetate, in amounts sufficient to be effective when located subcutaneously, was not effective when located intramesenterically. An effect was obtained, however, when larger amounts were implanted intramesenterically. This effect was less than that obtained from the subcutaneous pellets even though 3 times as much desoxycorticosterone acetate was used. The hormone absorbed from the intramesenteric pellets reaches the liver before entering the general circulation. That from the subcutaneous pellets directly enters the general circulation. It is therefore evident that the liver is responsible for the absolute or relative lack of effect of the intramesenteric pellets. Since the liver, then, completely inactivates a small amount of desoxycorticosterone acetate, as shown in *group* 3, but only partially inactivates a larger amount, as shown in *group* 4, it may be inferred that the liver is capable of handling only a certain amount of this substance and that amounts in excess of this limit will pass the liver without inactivation. This would explain why large amounts of desoxycorticosterone acetate must be given by mouth to be effective.

Kuizenga, Nelson and Cartland (13), in explanation of this diminished oral effectiveness of desoxycorticosterone acetate, suggested that it is partially destroyed in the gut since none was recovered in the feces after oral administration. This finding is not

necessarily proof that it is destroyed in the gut. If all the desoxycorticosterone administered orally were absorbed from the gut, there would also be none present in the feces. Eversole and Gaunt have supported the view that desoxycorticosterone is destroyed in the gut since they obtained no evidence of inactivation by the liver. It is unfortunate that no adequate explanation can be given for the disparity between their results and ours. The data of these authors have been published only in abstract form.

The proper location of pellets and the absence of vascular adhesions are extremely important factors in experiments of this type. Certain observations made in the course of experiments preliminary to those reported here have demonstrated their importance. Pellets of desoxycorticosterone acetate (*ca.* 25 mg.) were placed in the omental bursa of 11 adrenalectomized rats. Five of these animals were still alive 30 days later and 4 of the 5 had gained some weight (29, 31, 51 and 108 gm.). At autopsy careful search revealed no extraneous adrenal tissue, but in each of these animals the location of the pellet was such that the absorbed hormone was not exclusively conveyed through the portal system. In 3 animals there were adhesions to the body wall through which blood vessels from the pellet area could be traced. In another animal the pellet had migrated from the omental bursa and was found free in the scrotum. In the 5th animal the pellet had become adherent to the surface of the liver. In this location, at least a part of the absorbed hormone could probably pass directly into the efferent vessels of the liver, thus escaping the action of the liver cells. Biskind and Mark (4) have offered the same explanation for the incomplete inactivation of estrone and testosterone propionate when pellets of these substances are implanted directly into the liver.

SUMMARY AND CONCLUSIONS

Pellets of desoxycorticosterone acetate were implanted subcutaneously and intramesenterically in adrenalectomized animals. The majority of animals with subcutaneous pellets survived the experimental period and gained weight. The animals with intramesenteric pellets died at about the same time as the untreated adrenalectomized animals and showed a similar weight loss. These data demonstrate that the liver is able to inactivate desoxycorticosterone acetate. Pellets 3 times as large were then implanted intramesenterically in other experimental animals. These animals survived longer than the untreated controls and the animals with the smaller intramesenteric pellets. They showed a moderate weight gain although the per cent survival and weight gain both were less than in the group with the smaller subcutaneous pellets. These findings indicate that a portion of the hormone absorbed from the larger pellets escaped inactivation by the liver. It is therefore concluded that the liver can inactivate desoxycorticosterone acetate, but that this ability has quantitative limitations.

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ADRENAL AND PITUITARY WEIGHTS IN RATS WITH REDUCED GLUCOSE TOLERANCE

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PREVIOUS PAPERS from this laboratory have described a colony of rats of the Yale strain which, when compared with the Wistar strain, exhibited a higher fasting blood sugar, a lower tolerance for glucose, a more rapid rate of growth and a larger adult size, a longer and more irregular estrous cycle and a greater incidence of sterility (1–5). Subsequently, Orten and others (6, 7, 8) reported some similar traits in a related colony of the same strain. It appeared to us that the postulation of a hyperactivity of the anterior pituitary in the Yale strain offered the most satisfactory common denominator for the observed characteristics. Geiss (9) examined this hypothesis and showed that there was no difference between the differential cell counts of the

TABLE 1. PITUITARY AND ADRENAL WEIGHTS IN WISTAR AND YALE RATS

Strain and sex	Adrenal				Pituitary			
	A	b	S_y*	r†	A	b	S_y*	r†
Wistar, ♂	14.32	0.0412	2.51	0.82	3.056	0.0202	0.99	0.88
Yale, ♂	22.30	0.0474	4.54	0.79	3.530	0.0201	1.22	0.94
Wistar, ♀	6.84	0.1666	3.02	0.84	0.245	0.0518	1.38	0.90
Yale, ♀	1.50	0.1975	5.21	0.84	0.949	0.0403	1.54	0.85

*S_y = Standard error of the regression line.
†r = Coefficient of correlation.
Y = Adrenal or pituitary wt. in mg. X = Body weight in gm. Y = A + bX.

Males: Range in weight 140 to 440 grams

Wistar

Weight of adrenals in mg. = 14.32 + 0.0412 × body wt. in gm.
Weight of pituitary in mg. = 3.056 + 0.0202 × body wt. in gm.

Yale

Weight of adrenals in mg. = 22.30 + 0.0474 × body wt. in gm.
Weight of pituitary in mg. = 3.530 + 0.0201 × body wt. in gm.

Females: Range in weight 100 to 320 grams

Wistar

Weight of adrenals in mg. = 6.84 + 0.1666 × body wt. in gm.
Weight of pituitary in mg. = 0.245 + 0.0518 × body wt. in gm.

Yale

Weight of adrenals in mg. = 1.50 + 0.1975 × body wt. in gm.
Weight of pituitary in mg. = 0.949 + 0.0403 × body wt. in gm.

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anterior pituitary in males of the two strains matched according to age. However, the pituitaries from the Yale rats were larger than those from the corresponding Wistar animals. Since the Yale rats weigh more than the Wistar rats of the same age the significance of the observation on the size of the pituitaries would appear to depend upon the relation between pituitary weight and body weight. A report on this relation with similar data on the adrenals is the purpose of this paper.

METHODS

Males and females from the two strains were selected from that portion of the growth curve where one would expect a straight line relationship on the basis of

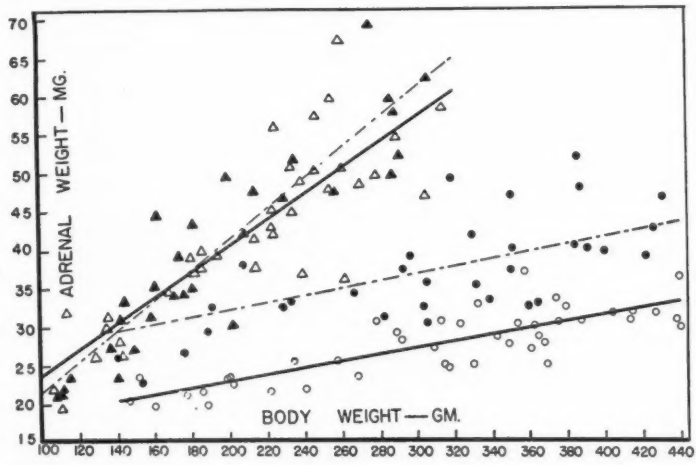

Fig. 1. Relationship between weight of adrenals and total body weight in rats. ○——Wistar males. ●- - - - -Yale males. △——Wistar females. ▲- - - - -Yale females.

Donaldson's organ to body weight data (10). The males ranged in weight from 140 to 440 gm. and the females from 100 to 320 gm. The rats were lightly anesthetized with ether, weighed to the nearest gram, and decapitated. Both adrenals with the surrounding tissue were rapidly removed to a sheet of unglazed paper, dissected, placed on a watch glass and quickly weighed to the nearest tenth of a milligram on a chainomatic balance. The skull was opened, the brain removed, the tissue around the pituitary stalk was dried with unglazed paper, the pituitary lifted out of the sella turcica and after quickly absorbing the adhering fluid with paper the gland was placed on a watch glass and weighed. The weight of each gland was plotted against the body weight of the rat and a straight line fitted by the method of least squares. The standard error and the coefficient of correlation were calculated for each line.

RESULTS AND DISCUSSION

The adrenal and pituitary weights of both sexes of the Wistar strain are lower than the values published by Donaldson (10) but the sex differential for the weights of the glands is of the same order as the differences recorded in Donaldson's curves. The discrepancy between our results and those previously published for the Wistar

strain may be real or it may be due to a more complete removal of the surrounding fluid in our experiments. The relationship between the adrenal and body weights are shown in table 1 and in figure 1. The difference between the regression lines for the adrenal weights of the Yale and Wistar males was greater than three times the standard error of Υ for the Wistar rats and less than three times but more than twice the standard error of Υ for the Yale rats. It is therefore highly probable that the greater ratio of adrenal weight to body weight in the Yale strain is significant. It is interesting to note that Yeakel and Rhoades (11) reported heavier adrenals in emotional than in non-emotional male rats but found no corresponding difference in the females. The

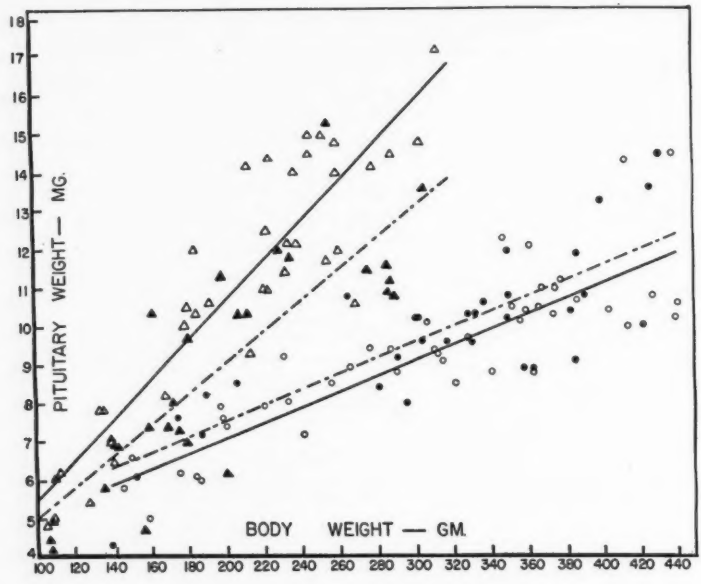

Fig. 2. Relationship between weight of pituitary and total body weight in rats. ○———— Wistar males. ●— — — —Yale males. △————Wistar females. ▲— — — —Yale females.

differences in the regression lines of adrenal weights for our Yale and Wistar females was less than a standard error of Υ of either strain and therefore not significant. There was no difference between the regression lines of the pituitaries from the males of the two strains. The regression lines for the pituitaries from the females in the two strains became increasingly divergent as the body weights increased (fig. 2). At 100 gm. there was no difference but at 300 gm. the difference between the two lines was almost exactly two times the standard error of Υ for the Wistar females but less than two times the standard error of Υ for the Yale females. This would indicate that Yale females of approximately 300 gm. of weight may have a significantly smaller pituitary than Wistar females of the same weight.

Any interpretation concerning the relation of the heavier adrenals of the Yale males to their decreased tolerance for glucose and other striking characteristics is rendered difficult by the absence of a similar difference in the females. In addition, the appearance in the females of a possible difference in the weights of the pituitaries, the opposite from that which one would expect, complicates the problem further.

SUMMARY

Using rats from the Yale and the Wistar strains, straight line relationships were found between adrenal weights and body weight and between pituitary weight and body weight in males ranging from 140 to 440 gm. and in females ranging from 100 to 320 gm.

Throughout the range in weight studied, the adrenals from the males of our Yale stock were significantly heavier than those from the males of our Wistar stock. Similar studies in the females revealed no difference between the strains.

In the males there was no difference between the ratio of pituitary weight to body weight in the two strains but in the females at approximately 300 gm. of body weight the ratio was possibly smaller in the Yale rats.

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RAT EAR AS A SITE FOR ADRENAL CORTICAL GRAFTS AND SUBSEQUENT EAR-ADRENALECTOMY[1]

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SITES MOST FAVORED for grafts of adrenal cortical tissue have been the ovaries, testes, kidneys and abdominal or dorsal body muscles (1, 2). Among the disadvantages of these locations are: *a*), lack of visibility; *b*), uncertainty as to whether life is being maintained by graft development and function or hypertrophy of accessory cortical tissue (or bits of cortex proper not removed at operation) or both; *c*), removal of the graft involves anesthesia and traumatic shock comparable to that encountered in ordinary adrenalectomy; and finally *d*), uncertainty of complete removal of the graft. Thus it appeared desirable to select a site that was visible and to develop a method of transplantation that subsequently would enable the investigator to remove all of the graft quickly, with little or no anesthetic, and with negligible trauma. The cortical deficiency so initiated would be practically a pure deficiency of the animal's own cortical hormones from the moment of its inception without the stimulus of trauma, to augment the deficiency and reciprocally influence the activity of any accessory cortical tissue that might be present.

The author is unaware of any reports of cortical transplants to the ears of rats or mice. Ovaries have been grafted in this location (3, 4, 5) and the success of those investigations led to the experiments reported here.

MATERIALS AND METHODS

Adrenalectomy was performed in one or two stages. Autoplastic transplants of ⅓ to ½ of an adrenal (92 rats) or a 'pressed out' piece of the capsule (12 rats) were placed near the tips of the pinnae. This was done by making a small slit in the skin of the dorsal side close to the base of the ear. Single homoplastic transplants were attempted in 11 normal rats 4 weeks prior to adrenalectomy and autoplastic transplants were made into the other ear. Some rats were gonadectomized at the time of the first stage of adrenalectomy. Some were given salt licks starting 1 to 18 days after the last transplant. The animal room temperature averaged 23 to 25° C. Rations were Wayne Dog Food Blox supplemented with lettuce.

All of the 25 females and most of the 79 males were Sprague-Dawley albino rats which experience a high mortality following adrenalectomy (6, 7). These animals were of 3 age groups: *a*), 43 to 112 days, *b*), 6 to 9 months and *c*), 19 months. Eleven experimental and control hooded males were from a colony that was sibling inbred for 29 generations. These rats experience a lower mortality and longer survival period following adrenalectomy.

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In addition to the above experimental transplant animals, a total of 40 control males and females were adrenalectomized as indicated in table 2. Mortality, body weight loss and survival data of another 65 rats previously reported (8) are included for comparison.

RESULTS

The condensed summary of part of the weight loss and survival data does not include results on 21 males that were 2-stage adrenalectomized and adrenal-to-ear

TABLE 1. BODY WEIGHT LOSS AND SURVIVAL OF ALBINO RATS FOLLOWING ADRENALECTOMY AND ADRENAL TRANSPLANTS INTO EARS, AND AFTER REMOVAL OF ADRENAL EAR GRAFTS

No. rats	NaCl licks[1]	% Wt. Change		Days of survival		Condition of transplant[2]	Remarks
		range	av.	range	av.		
		Died after transplants					
8♂		−22 to + 7	−11	6– 24	15	0–1	43–82 days old
14♂	yes	− 5 to −26	−18	6–173	59	(13)0–1, (1)4	adults, 9 castrates
13♀		−32 to +11	−11	11– 25	16	0–1	42–81 days old
7♀	yes	−17 to +11	−12	17–131	56	0–1	adults, 3 spayed
		Survived after transplants (from time of transplant to ear removal)					
13♂		+10 to +180	+106	24– 59	41	(2)0, (11)1–4	42–112 days old
10♂	yes	−12 to + 15	+ 2	41–173	93	(2)0, (8)2–4	3♂, NaCl stopped 35 days before ears removed. Adults
1♀			+12		24	4	
4♀	yes	+11 to +38	+25	195–210	202	(3)1, (1)4	2♀, NaCl stopped 60 days before ear removed. Adults
		Died after 'ear adrenalectomy' (from time of ear removal to death)					
11♂		−10 to −29	−21	5– 26	16	3–4	
2♀		−23 to −25	−24	21– 38	28	3–4	1 spayed
		Survived after ear removal (from time of ear removal to autopsy)					
12♂		−15 to +46	+12	25– 80	52	(4)0, (1)2, (7)4	
3♀		−2 to + 7	+ 3		22	1	1 spayed

[1] Salt licks placed in cages 1 to 18 days after adrenalectomy and transplants.
[2] Subjective evaluation of transplant size and development on a scale of 0 to 4.

Summary of experimental data

No. rats	NaCl licks	Postoperative Record			
		After transplant		After ear removal	
		died	survived	died	survived
21♂		8	13, 62%	8, 62%	5
24♂	yes	14	10, 42%	3, 30%	7
14♀		13	1, 7%	1	
11♀	yes	7	4, 36%	1, 25%	3

transplanted at 35 and 47 days of age. At the time of the second operation, the first autoplastic transplants appeared fair to good in 14 and poor in 8 rats. The animals were quartered in one large cage. All were dead within 4 to 13 (average 7.6) days after the second-stage operation and transplant. In many instances death was the result of cannibalism which centered about the head region and always included eating of the ears with their contained transplants. Apparently the rats were attracted by the slight drainage and small amount of coagulated blood at the site of the incision at the base of the ear. This cannibalism was not evident among the other young or older animals which were divided into smaller groups. However, it appears desirable to keep the animals in individual cages until the grafts become well established.

Male rats which died after transplants. Eight males did not have access to salt licks (table 1). Five were 43 and 3 were 82 days of age at the time the transplants were made. All died with what were judged to be poor or no 'takes' of the transplants. They lived more than 30% longer, on the average, than did adrenalectomized adult control animals (table 2). This is a minimal figure because it is well known that survival and weight losses of young rats are considerably lower than those of adults (9).

All of the 14 males that had access to salt licks after the transplants were operated in 2 stages with an interval of 2 weeks between operations. Autoplastic transplants,

TABLE 2. BODY WEIGHT LOSS AND SURVIVAL OF ADULT ALBINO RATS AFTER ADRENALECTOMY
Controls, no transplants or NaCl

No. rats	Type of operation	Mortality	Weight loss		Survival		Remarks
			Range	Av.	Range	Av.	
	stage	%	%	%	*days*		
11 ♀	1	100	8–23	16	5–22	12	Adr'y. at different times of cycle
20 ♀	2	100	11–32	23	9–23	13.9	Adr'y. in estrus
28 ♀[1]	2	96	9–28	22	4–30	14.4	Spayed 6–11 wk. before adr'y.
9 ♂	1	100	25–33	27.6	9–18	11.7	
22 ♂[1]	2	95	11–28	19.7	5–24	11.7	
150 ♂[1]	2	100	15–38	23	5–24	14.8	Castrated 6–7 wk. before adr'y.

[1] Previously reported (8) and included here for comparison.

first to one and then to the other ear were made at each operation. All of the animals were adults, 2 of them being of the oldest age group and 9 having been castrated at the time of the first operation. With the exception of one with a large graft that died and a 5% weight loss 85 days after the last operation, the transplants were very small if at all detectable. The weight losses averaged 18%; the survival periods 59 days. It is apparent that the availability of the salt licks markedly prolonged the survival periods but failed to prevent the usual body weight losses (table 2); there was not sufficient development of the transplants to maintain life.

Females which died after transplants. Eight of the 13 females not having access to salt licks were operated in 1 stage at 42 to 81 days of age and the remaining 5 had their 2-stage operations and transplants when they were 30 and 44 days old. Evidence that the cortical transplants prolonged the survival of these young rats is indicated by the 16-day average and the fact that none of them died before the 11th postoperative day. In all cases the transplants were either very small or the ears had become clear. The 7 2-stage operated young adult females receiving salt also had poor transplants, but they survived for prolonged periods just as the corresponding male rats. The weight losses of these females, however, were considerably below those of the males and were approximately 50% below the losses of the control adrenalectomized females.

Males surviving after transplants. Of the 13 males not receiving salt licks, 4 were operated in 1 stage at 42 to 82 days of age and 9 were operated in 2 stages with the adrenalectomy and transplant when they were 47 to 112 days old. An average of 41 days elapsed from the day of the last transplant to removal of the ear pinnae. By this time the rats had more than doubled their body weights and all but 2 had small to large grafts in one or both ears. These 13 surviving rats represented 62% of the animals in this age group; 8 rats, or 38%, died after the transplants as previously described.

Ten adult males had access to salt licks for 6 to 116 days (average 35) before removal of the ears. Their weight changes ranged from −12 to +15% (average +2%) during the 3 months from the time of receiving the transplants to the time of ear removal. Eight of these adults, including 2 old males, had medium to large grafts, but the ears of the remaining 2 animals had become clear. These 10 surviving rats represented 41% of the original group of 24 adult males receiving salt licks. The other 14 or 58% died after the transplants (table 1).

Females surviving after transplants. Only one, or 7%, of an original group of 14 young females which did not have access to salt licks survived after the cortical transplants. During the 24 days elapsing between the time of transplants and the time of ear removal it gained 12% in weight. This animal had first and second stage transplants subjectively rated as 2 and 4, respectively, on a scale of 0 to 4.

Of the 11 adult females receiving salt 4, or 36%, survived and gained an average of 25% in body weight during the 202 days elapsing from the time of receiving transplants to ear removal. Three had very small grafts, but one rated a classification of 4. The salt licks were removed from the cage 2 months before cutting off the pinnae of the ears.

Males which died after 'ear adrenalectomy.' The 11 males which died included 3 that had received salt licks to within 35 days of ear removal. This group of 11 constituted 48% of all the males, those previously receiving salt licks and those not, that survived after cortical autoplastic transplants to the ears. Their ear grafts were quite uniformly well developed and their weight losses after 'ear adrenalectomy' closely approximated those of the 2-stage, non-castrated controls (table 2). While the same was true of the range of their survival, the average was about 37% longer.

Females which died after 'ear adrenalectomy.' Two of a total of 5 females whose ears were removed died with weight losses very close to the average for the controls. One of these females had access to salt licks to within 2 months of ear removal. Though their survival periods far exceeded the average for the controls a larger number of experiments are necessary before conclusions may be drawn.

Rats surviving after ear removal. The 12 males which survived were autopsied at an average of 52 days after ear removal. Seven of these had access to salt licks to within 35 days of severing the ears. The ears of 2 of the 7 were clear and one had a graft rated as 2. The remaining 4 and 3 of the males which had not received NaCl previously had large grafts at the time of ear removal. All of the females which survived until autopsy on the 22nd day had small grafts. Cortical tissue was detectable in all but 2 of the males and females.

Hooded rats. Four of the 11 adult males were unoperated controls for body weight; 3 were 2-stage adrenalectomized controls without transplants; and 4 received autoplastic transplants following adrenalectomy in two stages. All of the rats had access to salt licks for 43 days from the time of the first stage operations. Two of the operated controls died 7 days after the salt licks were removed; their weight losses were 10 and 12%. One was autopsied after 30 days and had a weight loss of 21%. Cortical tissue was not detectable macroscopically. Of the 4 rats with transplants one died with a 26% weight loss 9 days after the second transplant. One of the 3 that survived after transplants had clear ears and possessed cortical tissue within the body at autopsy. The ears were removed from 2 rats with large grafts 43 days after the last transplant and 13 days after the salt licks were taken from the cages. At this time one was 8% above and the other was slightly below the weight level at the last transplant. The former was autopsied 80 days after ear removal when its weight loss was 24%. No cortical tissue was found. The other rat died with a weight loss of 18% 50 days after 'ear adrenalectomy.'

Condition of Grafts

The size of the grafts at the time of removal of the ears varied from small to large (fig. 1–4). Some of the small ones appeared ivory colored and poorly vascularized, whereas others were very red and diffuse. Without exception those with such poor grafts survived ear removal. This partly accounts for the large percentage of animals that survived after cutting off the ears; they possessed detectable cortical tissue within the body at the time of autopsy.

When changes in graft size were studied in relation to changes in body weight, the results were variable. Some of the rats possessed large ear grafts in the presence of cortical tissue within the body which was sufficient to maintain life and allow weight maintenance or gain following ear graft removal. On the other hand some of the adult rats experienced weight losses of as much as 24% from the weight level at ear removal; they subsequently regained some or all of this lost weight and lived until autopsy when cortical tissue was usually detectable in the adrenal regions. Such weight losses are in marked contrast with the author's experience that a loss of 14% of body weight following 1 or 2-stage adrenalectomy is a reliable criterion that a rat will die (8). It was observed that some of the grafts fluctuated in size with the passage of time. Some of the transplants were rated as *type* 0 for as long as 42 days, followed by a rating of 4 which may have been achieved in as little as 8 days. Sometimes this change was correlated with the opposite condition in the other ear graft. One male with a weight loss of 4% died in spite of the presence of a seemingly well developed graft.

A larger percentage of 2-stage adrenalectomized and adrenal-to-ear transplanted animals developed successful grafts than 1-stage operated and double transplanted rats. When the former procedure was followed, the second transplant to the remaining ear developed somewhat more frequently and usually became larger than the first transplant made at the first stage adrenalectomy.

Estrous Cycles

Those females with grafts sufficiently well developed to maintain life and approximately pre-operative weight continued normal estrous cycles. When removal of the grafts was followed by declining weight or eventual death, the rats became and remained diestrous. Additional experiments are necessary before quantitative comparisons with the vaginal changes following ordinary adrenalectomy may be made.

Homografts

Adult adrenal homografts into the ears of 53-day-old rats were large in 2 and present but small in 2 of 11 animals. Following double or second-stage adrenalectomy and unilateral autoplastic transplants to the other ear 28 days after the homoplastic transplants, 2 homografts became larger than the autografts. Removal of both ears in each of the 2 rats was followed by death in 17 days for one and survival with a weight gain of 11.5% by the time of autopsy on the 28th day for the other rat. A deep red body of cortical tissue was found near the left adrenal region. This animal was 4½ months old at the time of ear removal and had increased 11% in weight during the 24 days between the second-stage adrenalectomy (and autoplastic transplant) and ear removal. Its homograft appeared well developed at the time of the second adrenalectomy and subsequently became larger. After the ears were cut off there was not even a temporary decrease in body weight. This may indicate that the cortical tissue within the body was well developed while the ear grafts were present and they hypertrophied quickly following removal of the grafts as the result of over-stimulation, probably by way of the pituitary (12).

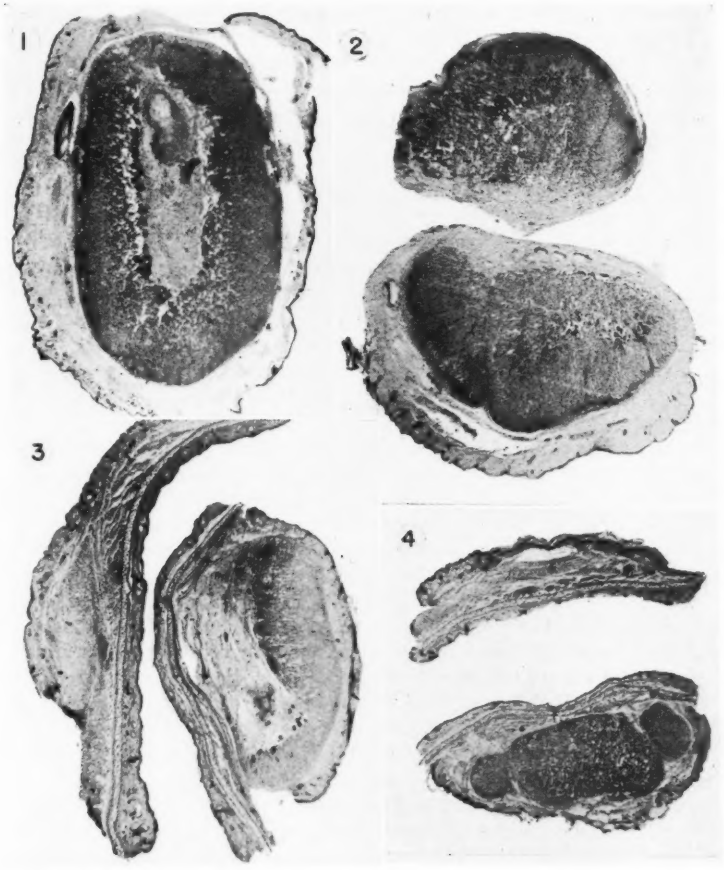

Fig. 1. RIGHT EAR ADRENAL CORTICAL AUTOGRAFT REMOVED FROM A MALE RAT 113 DAYS OLD, 30 DAYS AFTER 2ND STAGE ADRENALECTOMY AND TRANSPLANT TO RIGHT EAR. Transplant to left ear at 55 days age was unsuccessful. After removal of pinnae survival was 18 days, weight loss 16.2%. ×10. Fig. 2. RIGHT PEELED, AND LEFT EAR AUTOGRAFTS REMOVED FROM SPAYED RAT 7 MO. AFTER 2-STAGE ADRENALECTOMY. Salt lick accessible for 4½ months starting 12 days after last operation. After removal of grafts survival was 21 days, weight loss 23%. ×10. Fig. 3. RIGHT AUTOGRAFT AND LEFT HOMOGRAFT REMOVED FROM MALE RAT 112 DAYS AGE, 31 DAYS AFTER 1-STAGE ADRENALECTOMY AND AUTOPLASTIC TRANSPLANT TO RIGHT EAR. Homograft of ½ of an adult male adrenal was made at 53 days age. After removal of grafts survival was 23 days, weight loss 10%. ×10. Fig. 4. LEFT, BELOW, AUTOPLASTIC TRANSPLANT REMOVED FROM MALE RAT 93 DAYS AGE, 57 DAYS AFTER FIRST STAGE ADRENALECTOMY AND TRANSPLANT TO LEFT EAR. Second stage adrenalectomy and transplant to right ear was performed at 63 days age. Right ear graft is not present at level shown, but was approximately ⅓ size of left graft. After removal of grafts survival was 11 days, weight loss 19%. ×10.

DISCUSSION

Comparison of the summary of table 1 and the mortality percentages in table 2 appears to indicate that far more rats possess accessory cortical tissue than might be concluded from mortality after complete adrenalectomy. Variations in the amount of this tissue may partly explain the wide range of survival time following adrenal

removal. The observation that a number of transplants became and remained clear (*type 0*) for several weeks after transplantation and subsequently, within 8 to 10 days, became large *type 4* grafts indicates the unusual potentialities of microscopic groups of extracapsular cortex-like cells. If the rats which die after adrenalectomy and autoplastic transplants are disregarded, it becomes apparent that the number of survivors (following graft removal) increases greatly if the animals are maintained for some time by cortical transplants. Under these conditions accessory cortical tissue which was probably insufficient at the onset to maintain life has a chance to develop enough to serve as a substitute for the gland.

The large percentage of rats which develop cortical tissue within the body sufficient to maintain life in spite of an unsuccessful transplant or removal of a successful one confirms Gaunt's reports (10, 11) that rats which ordinarily have a 95% mortality after adrenalectomy may show a 50% mortality after cortin or salt therapy has been stopped several weeks after the operation. The transplants, successful or not, may likewise allow time for development of accessory cortical tissue.

The 37% longer average survival time of the 11 males which died after 'ear adrenalectomy' implies that even a 2-stage operative procedure involves factors which reduce the average survival time (and thus increases the degree and rate of cortical insufficiency) by at least 27%. Since the majority of these males were only 2½ to 3 months of age at the time of graft removal, the difference appears more likely to approach 40 or 50% in older animals. If anesthetics, trauma, and the uncertain rôle of accessory cortical tissue are to be largely eliminated as factors in experiments on adrenalectomized rats, a preferred method would be to use only those rats which survive and develop large cortical grafts in the ear(s); control experiments could be performed on some of these and on normal rats. After 'ear adrenalectomy' the experiments should be carried through with all of the ear-graft animals and at the end of each experiment the animals should be allowed to lapse into cortical insufficiency. The observations on those rats whose survival exceeds certain time limits should be discarded or considered in a separate category (since many may be chronically insufficient) because they would demonstrate the presence of cortical tissue. While this method would require more time and animals than would the alternative of cortical extract injections and later cessation of such treatment, the continuous secretion of cortical compounds by the animal's own cortical grafts appears more likely to approximate normal physiological conditions.

The highest percentage (52%) of successful transplants was obtained with males which did not have access to salt licks. This figure is considerably below the 95% for intramuscular transplants reported by Wyman and tum Suden (12, 13) who have accumulated 430 cases. Two factors that may partly account for the difference appear to be the cooler temperature of the ear (14) and the extent of the capillary bed. Both factors appear to be easily controlled, because experiments in progress involving increased environmental temperature and especially stimulation of the pinnae before and after transplantation appear to increase the likelihood of successful development of autoplastic transplants.

The pinnae of the ears offer many advantages over the sites usually employed for adrenal cortical transplants. Most important and obvious among these advantages are visibility, and as a result, the certain removal of all of the transplant quickly and with negligible trauma. In this location it is possible to make daily observations of changes in graft size and color and to correlate them with changes in body weight, the presence and extent of accessory cortical tissue, and experimental procedures such as dietary factors, inanition, shock, modified environmental temperature, hormone administration and castration.

SUMMARY

The use of the ear as a site for adrenal cortical transplants and subsequent graft removal was studied in 102 adrenalectomized albino rats. Forty adrenalectomized rats served as controls.

The transplants were successful in about ½ of the males as contrasted with ⅓ of the males that had access to salt licks after the transplants. Those of the latter group that died experienced markedly prolonged survival periods. Forty-eight per cent died following graft removal when none of them were receiving salt licks; most of the others possessed detectable cortical tissue within the body. Those that died experienced weight loss and a survival range similar to those of the controls, but they lived 37% longer on the average.

Only 7% of the females survived with well developed grafts as compared with 36% of the females receiving salt licks after the transplants. After the ears containing grafts were removed, 40% of all of the survivors of both groups died after survival periods which exceeded those of the controls.

The size of the grafts within the ears fluctuated with time and this was correlated with corresponding body weight changes in most cases. The exceptions appeared to result from the activity of accessory cortical tissue within the body. The decreased mortality after ear graft removal indicates that far more rats possess accessory cortical tissue than might be concluded from mortality after 1 or 2-stage adrenalectomy.

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INFLUENCE OF BLOOD EXTRACTS FROM NORMAL, GOITROUS, AND DIABETIC PERSONS ON THE HEART RATE OF THE THYROIDECTOMIZED RAT

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IN AN INVESTIGATION of the relation between the faculty of normal and of pathological thyroids to increase the metabolism and to accelerate the heart in thyroidectomized rats it was observed that healthy glands in doses having a conspicuous effect on metabolism had only an insignificant action on the heart rate. The thyroid from a patient with Grave's disease who had not received iodine preceding operation behaved quite similarly (1). However, thyroids from thyrotoxic patients who had received iodine treatment showed a considerably enhanced action on the heart as compared with that on metabolism.

If normal animals were treated with iodine for some weeks, the heart-stimulating capacity of their thyroids, insignificant before, was brought to high levels (2).

It is known that in hyperthyroidism the metabolic hormone of the thyroid is released at an increased rate into the blood stream. This is manifest by the higher average blood iodine values in the hyperthyroid patient as compared with that of normal subjects (3). Iodine treatment leads to an accumulation of the hormone in the gland with an increase of its colloid content. A simultaneous drop of the hormone circulating in the blood cannot be demonstrated by the same chemical method, since continued absorption of iodine from the intestine maintains a high blood iodine level.

An analogous problem presented itself with reference to the heart-stimulating factor, namely the question of its presence or absence in the circulating blood in normal and pathological conditions and the possible influence of iodine treatment on its blood level. Since it has been found that the substance responsible for the action on the rat's heart can be extracted from dried thyroid glands with certain organic solvents, a possible method for such an investigation was given.

METHOD

Citrated blood, obtained from the patients in the usual way, was placed in flat dishes in thin layers and dried in a stream of warm air. The air current was turned off as soon as the drying blood began to break into small scales, since they are easily blown off. The drying process was finished in the open air, the substance being protected against contamination by dust. The dry blood was ground to pass through a sieve, mesh 40, and extracted with warm alcohol in a Soxhlet apparatus for 2 days. The extract obtained by evaporation of the alcohol was suspended in a small quantity of water and aliquot quantities fed to the rats by stomach tube on 3 consecutive days.

The method of testing has been described in previous publications (4).

The quantities of blood necessary for a reliable test had to be determined by trial.

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TABLE 1. EFFECT OF BLOOD FROM CONTROLS, NORMAL AND DIABETIC PERSONS ON RATS

Case	Date	Age yr.	Wt. lb.	Pulse rate	State of health	Tests on rats		
						cc. blood/ rat	Change in B.M.R.	Change in heart rate
2, B.A. ♂	10/22	34	175	68	Healthy	11	+ 8	0
						23	+ 5	0
3 ♀	10/22	55		58	Cerebral thrombosis. Healthy before	36	− 4	0
7, A.R. ♂	11/28	60	160	68	Diabetes	25	+20	+ 85
	12/12					15	0	+ 10
						28	+ 4	+ 46
	12/17					12½	+ 3	+105
						12½	+ 7	+102
	1/ 8					12½	+30	+ 54
						12½	+16	+ 50
19, D.U. ♀	1/20	65	169	90	Diabetes. Blood sugar 350 mg.%	11	+ 8	+ 2
						11	− 4	0
20, R.B. ♂	1/20	49		72	Diabetes. Blood sugar 280 mg.%	11½	+ 8	+ 4
						11½	+20	0
21, A.K. ♀	1/20	60		78	Diabetes. Blood sugar 250 mg.%	12	− 7	+ 5
						12	+ 5	+ 20
22, M.A. ♂	1/28			64	Diabetes. Blood sugar 160 mg.%	13	+ 7	+ 18
						13	0	+ 22
26, G.B. ♀	2/ 3	51	145	62	Diabetes. Cholecystectomy. Ovariectomy	28	− 3	+ 21
27, T.D. ♀	2/ 3	56	130	74	Diabetes; 40 U insulin	27	− 4	+ 8
28, H.K. ♀	2/ 3	51	105	90	Diabetes; 40 U insulin	27	0	0
30, T.G. ♂	2/ 9	60	186	74	Diabetes; Insulin	18	+20	+ 31

A negative result becomes more convincing the larger the dose used has been and positive results carry more weight if elicited by small quantities of material.

In view of the unavoidable variations inherent in all biological tests, a consideration of the limits of spontaneous variations usually observed in metabolic tests on thyroidectomized rats appears necessary. Increases of 10% in metabolic rate must be considered as lying within the limits of error. In view of the few animals used on each test, the results in this range are not suited for statistical interpretation, even if the change in the various test animals should closely agree. Changes in metabolic responses up to 10%, therefore, are considered as negative. Stimulations which give between 10 and 20% increase correspond, in graphic presentation, to the steep ascending branch of the logarithmic curve, where the margin of error is large. Consequently, not much importance should be attributed to small differences and any response between 10 and 20% should be considered as positive, but low, without quantitative evaluation. Higher values indicate significant metabolic stimulation.

The difference in heart rate between the normal and thyroidectomized rat averages 100 beats per minute. However, it has been observed that by feeding the heart-

TABLE 2. EFFECT OF BLOOD FROM CASES OF TOXIC HYPERTHYROIDISM, UNTREATED, ON RATS

Case	Date	Age yr.	Wt. lb.	Exophthalmos	Tremor	Insomnia	Pulse rate	B.M.R.	Diagnosis	Test on rats: cc. blood/rat	Test on rats: Change in B.M.R.	Test on rats: Change in heart rate
				Clinical Data								
1, V. ♂	10/ 9	23	146	+	+	+	90	+ 6	Thyroid diffusely enlarged for 8 mo.; symptoms for 10 mo.	55	0	+102
6, J.M. ♀	11/28	22	189				102	+42	Toxic adenoma	27	+ 6	+ 71
8, M.R. ♀	12/ 4	41	109				84	+68	Toxic diffuse goiter[1]	12	0	+ 84
										22	− 1	+ 61
9, L.D. ♀	12/ 2	24	121				88	+54	Toxic diffuse goiter[1]	24	+19	+ 69
	12/18	Thyroidectomy performed; no record of preceding iodine treatment available										
	2/ 4						72	+21		14	− 4	+ 14
										14	0	+ 21
10, G.H. ♀	12/13	64	140		+	+	84	+20	Thyroidectomy 15 yr. before. Recurrent Grave's disease. Diabetes	15	+ 4	+ 79
										15	+ 6	+ 82
										15	+10	+ 75
	12/ 7									12	+ 4	+ 96
										12	0	+ 94
11, ♀	11/28	24		+	+	+	110	+64	Grave's disease[1]	10	− 3	+ 65
										10	+ 8	+ 66
23, L.B. ♀	1/28	39	229	+	+	+	96	+46	Hyperthyroid (Thyrocardiac)	12	0	+ 56
										12	0	+ 67
25, M.C. ♀	1/28	53	129	+	+	+	84	+47	Toxic diffuse goiter[1]	13	+ 5	+ 46
										26	− 4	+ 64
13, M.G. ♀	1/ 8	24	113				96	+14	Toxic nodular goiter[1]	14	0	+ 3
										14	− 1	0
	1/11	Iodine treatment started										
	1/15									16	+ 3	+ 6
										16	− 3	+ 4
										12	+ 2	+ 5
										12	+ 2	+ 5
37, H.B. ♀	3/31	44	153				84		Toxic nodular goiter[1]	14	+ 4	+ 2
										14	+ 4	+ 1

[1] See report in text.

stimulating extract prepared from desiccated commercial thyroid which is free of the metabolic hormone to thyroidectomized rats, the heart rate is not always increased the 100 beats per minute and it almost never exceeds 100 regardless of the quantity of material given as treatment. Increases of 60 beats per minute are quite common. Spontaneous variations practically never exceed an increase of 30 beats. Therefore,

TABLE 3. EFFECT OF BLOOD FROM CASES OF TOXIC GOITER, IODINE TREATED, ON RATS

Clinical Data

Case	Date	Age yr.	Wt. lb.	Exophthalmos	Tremor	Insomnia	Pulse rate	B.M.R.	Diagnosis	Test on rats		
										cc. blood/ rat	Change in B.M.R.	Change in heart rate
14, M.J. ♀	1/ 7	51	100				160		Hyperthyroidism with heart disease. Iodine since 12/21	13 13	+ 7 + 2	+ 24 + 13
15, M.I. ♀	1/ 8	66	96				100	+81	Diffuse toxic goiter. Bilateral pole ligation. Heart disease. Iodine since 11/11	18 13	+ 6 − 1	+ 7 + 16
16, W.L. ♀	12/ 9	56	196				109	+67	Toxic nodular goiter. Iodine since 12/9			
	1/ 7						84	+18		20 14	+ 1 + 6	+ 5 − 2
18, O.Z. ♀	1/10	32	148	+	+	+	108	+78	Toxic diffuse goiter.[1] Iodine treatment started 1/13			
	1/16			+	+	+				14 14	+ 5 − 1	+ 55 + 60
	1/29								Thyroidectomy			
	2/ 4						74			12½ 12½	+ 5 − 2	+ 4 + 13
32, C.M. ♀	2/20	43	170	No	+	+	80	+26	Diffuse toxic goiter.[1] Iodine since 2/13	15 15	+26 +20	+ 72 +103
	3/ 6								Thyroidectomy on same day as blood sample	12 12	+ 3 + 4	0 + 2
35, P.R. ♀	2/20	49	120				120	+62	Toxic diffuse goiter.[1] Iodine 2/20	12 12	+ 9 − 2	+ 72 + 60
	3/ 6							+29		15 15	+ 7 − 6	+ 3 − 2
31, C.M. ♀	2/20	46	94	(+)	No	No	128	+ 3	Non-toxic nodular goiter.[1] Iodine since 2/19	15 15	+15 +23	+ 99 + 96
	3/ 3								Thyroidectomy			
	3/13						80			13 13	− 4 + 5	+ 3 + 6

[1] See report in text.

it is safe to assume that variations of 30 beats or less must be considered as negative. We consider responses of 50 beats and more per minute as positive. Questionable figures between 30 and 50 rarely occur, which provides a clean division between positive and negative results. The data obtained are presented in tables 1 to 4.

TABLE 4. EFFECT OF BLOOD FROM CASES OF NON-TOXIC THYROID DISEASE AND DUBIOUS CASES ON RATS

Case	Date	Age yr.	Wt. lb.	Pulse rate	B.M.R.	Diagnosis	Test on rats: cc. blood/rat	Test on rats: Change in B.M.R.	Test on rats: Change in heart rate
						Clinical Data			
29, G.C. ♀	2/ 4	42	133	112	+34	Thyroidectomy 5 yr. before. Relapse. No tremor or exophthalmos	26	− 6	+10
34, E.K. ♀	2/20	51				Non-toxic nodular goiter.[1] Tremor. Insomnia	15	+ 9	+22
							15	0	+53
5, E.W. ♀	10/31	38	165	85	+31	Neurasthenia and hysteria. No thyroid enlargement	26	+ 8	0
					+23				
4, D.G. ♀	10/ 3		125			Non-toxic adenoma. Mass of 1″ in region of isthmus	27	− 4	0
12, N.O. ♀	12/23	24	118	99[2]	+20[2]	Post-thyroidectomy. Cachexia. Myxedema	15	+ 5	+30
							15	0	+29
17, A.M. ♀	1/16	61	166	72	− 4	Non-toxic nodular goiter[1]	14	+15	+12
							14	+17	+19
33, M.R. ♀	2/20	26				Diffuse non-toxic goiter	14	− 5	+ 9
							14	− 2	+ 9
24, A.C. ♀	1/24	42	199	88		Non-toxic hyperthyroidism	13	− 5	+23
							13	0	+27

[1] See report in text.
[2] Receiving 5 grains of thyroid daily.

DISCUSSION

In table 1 the only subjects which were actually normal from an endocrine standpoint were *cases* 2 and 3. The blood of both, even at large doses, produced neither metabolic nor heart rate changes in the test.

Diabetics were chosen as controls since such blood was easily available to us. Seven of these justified their use as negative controls. *Case* 30 might be regarded as approaching the borderline, although essentially on the negative side. *Case* 7 almost consistently gave positive tests in increasing the heart count and repeated increases in metabolism. This patient had no other endocrine disturbance but diabetes and gave a normal response to insulin. There is no means of explaining the abnormal responses in this case, which cannot be attributed to an experimental error since several repetitions of the test confirmed the first positive result.

The second table contains data from 8 cases of frank hyperthyroidism. The tests uniformly showed a strong action on the rat's heart but an essentially negative metabolic assay.

The last two cases, 13 and 37, did not give a positive reading in either respect. Both cases were finally diagnosed as 'toxic nodular goiter'; exophthalmos, tremor, and insomnia were not observed in these cases; one showed a moderately increased metabolic rate of +14. Nevertheless, thyroidectomy was performed in both cases. Whether nodular goiter is physiologically different from other types of hyperthyroidism, or the toxicity in these two cases happened to be of an exceptionally low order remains a moot question.

The data in table 3 illustrate the influence of iodine in hyperthyroid patients. The first 3 had received treatment when the first blood sample was taken. No action on the rat's heart was observed in the tests. *Cases 32* and *35* gave a positive test before treatment and subsequently became negative as iodine was given. An iodinization period of 7 days in *case 32* and of 3 days in *case 18* did not bring about a change in the reaction. The next blood sample of *case 18* was taken several days after thyroidectomy. The negative result in this case might be attributed to removal of the thyroid as well as to the iodine treatment.

Case 31 was diagnosed as having non-toxic goiter. A slight exophthalmos and pulse rate of 128, however, are not quite in accord with this diagnosis. The patient initially showed a normal metabolic rate and a decidedly increased pulse rate which subsided under iodine treatment. The test on the rat would suggest a toxicity with the usual subsidence following iodine.

In table 4 it is interesting to note that the relapse of *case 29* after thyroidectomy gave a negative test in distinction from *case 10* in table 2. The clinical diagnosis and the test in the rats remained dubious in *case 34*. *Case 5* was tested only on account of an initial erroneous diagnosis of hyperthyroidism. In this case the negative test preceded the correction of diagnosis.

The tables contain only a few values for increased heart rate which are difficult to classify as a positive or negative response. It is also apparent that a correlation between size of dose and increase in heart rate seems to be entirely lacking. In *case 8*, table 2, in which the amount of material was almost doubled, there was a lower response. This absence of a quantitative relationship between treatment and effect has been observed also with extracts prepared from thyroid material. The impression is gained that one is dealing with an all or nothing reaction.

The question may be raised concerning the significance of the increase in metabolic rate obtained in various cases. If the metabolically active iodine in the blood were present exclusively as globulin compound, its insolubility in alcohol should prevent if from passing into the extract. However, after hydrolysis of the globulin the thyroxin component is soluble in alcohol and therefore the extract might contain some of the metabolic principle if hydrolysis should take place during the process of extraction.

From the figures for blood iodine in various conditions, as reported in the literature, it can be said that the iodine content in the quantities of blood as used, is normally below the threshold that elicits a positive response in the thyroidectomized rat. The figures reported for pathological conditions frequently approach or even exceed that limit. The extract used, of course, should contain only the fraction called by Salter "anorganische" iodine. However, it is possible that in the prolonged extraction used for these tests relatively large amounts of the active iodine were extracted.

The occasional metabolic responses obtained in the experiments presented are too erratic to permit even tentative interpretations. The work was done primarily with reference to the heart-stimulating factor, and in this respect the results seem to be significant, within the limits of this research, indicating that in toxic hyperthyroid conditions a substance circulated in the blood that stimulates the heart rate in the thyroidectomized rat. This substance was found to be absent or present in smaller quantities in *a*) normal persons, *b*) in patients with non-toxic thyroid disease, and, as a rule, *c*) in non-complicated diabetes. Treatment of the thyrotoxic patient with iodine causes the substance to disappear from the blood stream.

The physiological significance of the heart-stimulating substance is still obscure. The lack of proportionality between dose and reaction makes it at this stage of our

investigation still more difficult to decide whether one is dealing with an accessory thyroid hormone or a product of a pathological function of the gland.

The observation of its accumulation in the normal thyroid after iodine treatment does not necessarily contradict the assumption that one is dealing with a pathological product, since the flooding of the gland with iodine is likely to produce physiological changes. It will be necessary to extend the study in two directions—isolation and purification of the problematic substance responsible for these phenomena and elucidation of its presence or absence in the various stages of histologically different thyroids.

SUMMARY

There seems to be evidence that in cases of untreated toxic goiter a substance is circulating in the blood which stimulates the heart of the thyroidectomized rat. This substance is not present in quantities sufficient to be detected in normal persons, diabetics or in cases of non-toxic hyperthyroidism; it disappears in Grave's disease under the influence of iodine treatment.

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PROTOCOLS

Case 8, M. R. Specimen of thyroid grayish purple in color; surface roughly nodular. Section revealed a homogeneous somewhat greasy grayish tan structure. *Microscopic examination* showed marked hyperplasia of the acini, the acini being filled with a dark pink staining colloid. They are lined by flattened epithelium and many show marked proliferation of the epithelium into the lumina. The interstitial tissue is scanty and shows occasional hypertrophied lymph follicles. *Diagnosis:* toxic diffuse goiter.

Case 9, L. D. Specimen of a thyroid grayish purple in color. Section reveals a homogeneous grayish tan, lobulated structure. *Microscopic examination* showed marked dilation of acini which are lined by flattened epithelium and filled with a dark pink staining colloid. Many of the acini show some papillary proliferation of the epithelium into the lumina. The interstitial tissue is scanty and shows occasional hypertrophied lymph follicles. *Diagnosis:* toxic diffuse goiter.

Case 11. This blood specimen was obtained through the courtesy of Dr. W. T. Salter, Boston City Hospital. The blood was taken from a 24 year-old-woman with Grave's disease of 4 months' duration. She had the classical symptoms of nervousness, tremor, goiter and exophthalmos, but had never noted palpitation. She demonstrated bilateral exophthalmos, a tachycardia of 110 per minute and a large, firm goiter. Her initial B.M.R. was +64% which fell to −19% with iodine therapy. The goiter was removed, proving to be a typical hyperplastic gland, and the patient's postoperative course has been entirely satisfactory.

Case 13, M. G. Specimen consists of 2 pieces of thyroid gland tissue. *Microscopic examination* showed marked hyperplasia of the glandular structures, glands being lined by flattened epithelium and filled with a light pink staining colloid. Many of the acini show some papillary proliferation of the epithelial lining in the lumina. Interstitial tissue is increased and shows occasional hypertrophied lymph follicles. *Diagnosis:* toxic nodular goiter.

Case 17, A. M. Specimen consists of a lobe of thyroid gland. There appears to be a large cystic cavity containing some grayish tan material. *Microscopic examination* showed a marked hyperplasia of the acini, acini being lined by flattened epithelium and filled with a light pink staining colloid. Many of the acini show vacuolization of the colloid. Interstitial tissue is somewhat increased and areas of round cell infiltration are noted in the interstitial tissue. No malignancy or tumor seen. *Diagnosis:* non-toxic nodular goiter.

Case 18, O. Z. Specimen consists of 2 pieces of thyroid tissue, each 5½×3½ cm. Cut section is homogeneous grayish tan. *Microscopic examination* showed hyperplasia of the acini, acini being filled with a light pink staining colloid and lined by a flattened epithelium. Many of the acini show papillary proliferation into the lumen and many show entire obliteration of the lumen. Hypertrophied lymph follicles are noted in the interstitial tissue. *Diagnosis:* toxic diffuse goiter.

Case 25, M. C. The right lobe weighed 12 gm. and measured 6×4×2 cm. On section shows hyperplasia with colloid degeneration; fibrous and hard throughout. The left lobe weighed 10 gm. and measured 5×3×1½ cm. On section, showed hyperplasia with colloid degeneration. *Diagnosis:* toxic diffuse goiter.

Case 31, C. M. Specimen consists of a lobe of the thyroid gland tremendously enlarged, measuring 11×6¼ cm. It is grayish tan in color. There is an area of calcification under the capsule. *Microscopic*

examination showed marked hyperplasia of the acini, acini being lined by flattened epithelium and filled with a dark staining colloid. Interstitial tissue is markedly increased and shows areas of calcification and in some areas definite adenomatous-like structures. There is no evidence of malignancy. *Diagnosis:* non-toxic nodular goiter.

Case 32, *C. M.* Specimen of thyroid gland, consisting of 2 lobes. Cut section is grayish tan in color and nodular in appearance. *Microscopic examination* showed marked hyperplasia of the glandular structures, the acini being lined by flattened epithelium and filled with a dark pink staining colloid. Lining cells of many of the acini show papillary proliferation into the lumina. Many hypertrophied lymph follicles are also noted. Interstitial tissue is scanty. There is no histologic evidence of malignancy. *Diagnosis:* toxic diffuse goiter.

Case 34, *E. K.* Specimen consists of a lobe of the thyroid gland, greatly enlarged, and measuring $7\frac{1}{2} \times 4$ cm. It is nodular in appearance, grayish tan and dark brown in color. *Microscopic examination* showed the acini are markedly dilated and lined by flattened epithelium and filled with a light pink staining colloid. Interstitial tissue is markedly increased and shows areas of infiltration with small round cells. There is no histologic evidence of malignancy. *Diagnosis:* non-toxic nodular goiter.

Case 35 *P. R.* Specimen consists of 2 lobes of the thyroid gland. They measure approximately 4×3 cm. They are grayish brown in color. One piece from each lobe referred for microscopic examination. *Microscopic examination* showed a marked hyperplasia of the glandular structures. They are filled with a light pink staining colloid and lined by flattened epithelium. Many of the acini show papillary proliferation of the lining cells into the lumina. Interstitial tissue is scanty and shows occasional hypertrophied lymph follicles. *Diagnosis:* toxic diffuse goiter.

Case 37, *H. B.* Specimen consists of two masses. The largest measures $7\frac{1}{2}$ cm. in its greatest length and $8\frac{1}{2}$ cm. in its greatest width. The mass contains two lobes of the thyroid gland. The surface is of a grayish white brown mottled appearance. It is firm throughout and somewhat resilient and there are several polypoid projections on one lobe. This thyroid on section reveals a grayish tan brown mottled appearance with a friable soft substance. Several sections were referred for microscopic examination. *Microscopic examination* showed a marked hyperplasia of the glandular structures, acini being filled with a light pink staining colloid, many acini show a papillary proliferation of the epithelium in the lumina. Interstitial tissue is markedly increased and in areas form adenomatous-like structures. Interstitial hemorrhages are noted occasionally in the gland. There are also noted areas of calcification on the interstitial tissue. *Diagnosis:* toxic nodular goiter.

EFFECT OF THE SERUM OF PATIENTS WITH GRAVES' DISEASE, MYXEDEMA AND ACROMEGALY UPON THE OXYGEN CONSUMPTION (QO_2) OF GUINEA PIG THYROID

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IN A PREVIOUS PAPER (1) it was shown that the action of human serum upon the oxygen absorption of guinea pig thyroid tissue is determined by the algebraic sum of the concentration of thyroid hormone and thyrotropic hormone contained therein. Thyrotropic hormone raises QO_2, thyroid hormone lowers it. The hormones can be added to serum in such quantities that their actions completely cancel one another.

In the present paper such an approach has been utilized for the study of the hormone balance in clinical disease.

The determination of oxygen consumption (QO_2) of thyroid tissue exposed to patients' serum was by the standard technique of Warburg. The technique of preparation of tissues and sera was described in the first paper (1).

Observations were made in 5 cases of acromegaly, 4 of classic Graves' disease, 1 of the type of Graves' disease with dissociation of ophthalmopathy and thyrotoxicosis (2), 2 of untreated myxedema and 1 of treated myxedema (these 3 were all primary thyroid myxedema).

In figure 1 are shown the QO_2% variations from control serum in all these cases, together with the values for B.M.R.

The serum of all of the acromegalics stimulated the guinea pig thyroids, producing elevations of QO_2 of 33, 45, 37, 13 and 32%. The B.M.R. of 3 of these patients, however, showed no elevation, they being in the burned-out stage of the disease; that of 2 in an active phase showed +15 and +25

The sera of 3 of the patients with classic Graves' disease depressed the QO_2 of guinea pig thyroids to −15, −22, −28 and −44%; that of 1 caused an elevation. The B.M.R. of all of these was, of course, elevated. The serum of the first and the last patient of *group 2* in figure 1, when tested again after subtotal thyroidectomy showed values of +8 and +4, with a B.M.R. of −5 and +10, respectively, *group* 3, figure 1.

The serum of patients with the special ophthalmic type of Graves' disease caused an elevation in QO_2 of 34 and 21% in 1 case, and of 32, 20 and 15% in 3 other cases. The B.M.R. in the 1st case was +19 on 1 occasion and +3 on another; 2 other cases were 0; and the last one was +10.

The serum of patients with untreated myxedema caused elevation in QO_2 of 57, 48, 26 and 35%, the corresponding B.M.R. being −40, −38, −23 and −38.

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I wish to thank Dr. J. H. Means for the facilities he has given me in his laboratory and for his supervision of my work,

The serum of 4 treated myxedema patients showed B.M.R. −10, $\mathcal{Q}O_2$ −34; B.M.R. −6, $\mathcal{Q}O_2$ −10; B.M.R. −22, $\mathcal{Q}O_2$ +10 (these last 2 patients are the same ones who showed, previous to treatment, a $\mathcal{Q}O_2$ of +57 and +48, respectively), B.M.R. −10, $\mathcal{Q}O_2$ +4. The serum of 2 cases of pituitary myxedema, with a B.M.R.

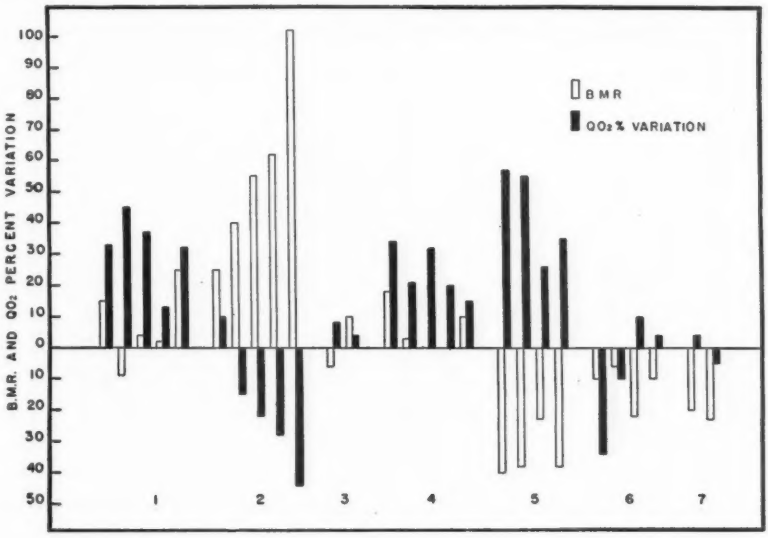

Fig. 1. B.M.R. of patients (white columns) with, *group 1*, acromegaly. *group 2*, classic Graves' disease, untreated. *group 3*, classic Graves' disease after thyroidectomy. *group 4*, Graves' disease with dissociated ophthalmopathy. *group 5*, Myxedema, untreated. *group 6*, Myxedema, treated. *group 7*, Hypopituitarism.

The $\mathcal{Q}O_2$ of guinea pig thyroid immersed in patient's serum is shown in each case by a black column.

of −20 and −23, respectively, showed no considerable difference with the normal controls, $\mathcal{Q}O_2$ +4 and −5, respectively.

DISCUSSION

It is believed that the mechanism producing these results in disease is similar to that involved in the experiments previously reported in which known quantities of the hormones were added to normal serum. The $\mathcal{Q}O_2$ variation of guinea pig thyroid caused by the sera of patients must be regarded as the result (algebraic sum) of the opposite actions of the 2 hormones present in them. The values obtained indicate an excess of thyrotropic hormone activity over thyroid hormone activity in acromegaly, untreated myxedema, and in the special ophthalmic type of Graves' disease. In classic Graves' disease, on the other hand in 3 of 4 cases the values indicated an excess of thyroid hormone action over that of thyrotropic hormone. The same is true in treated myxedema.

In an attempt to arrive at the significance of these data a schema has been prepared of the sort used by Fraser, Albright and Smith (3) to portray the opposing influences which affect blood sugar.

In figure 2 the white arrows represent circulating thyroid hormone and the black arrows thyrotropic hormone. The respective actions, stimulating or depressing the

$\mathcal{Q}O_2$ of thyroid tissue, are expressed by the direction of the arrows. The black dots between each pair of arrows represent the $\mathcal{Q}O_2$ of the thyroids tested in such sera.

For the normal subject in whose blood stream it may be assumed there is neither an excess nor deficiency of either hormone, that is to say in whom there is stable hormonic equilibrium, arrows of equal length may be drawn as in figure 2, *A*.

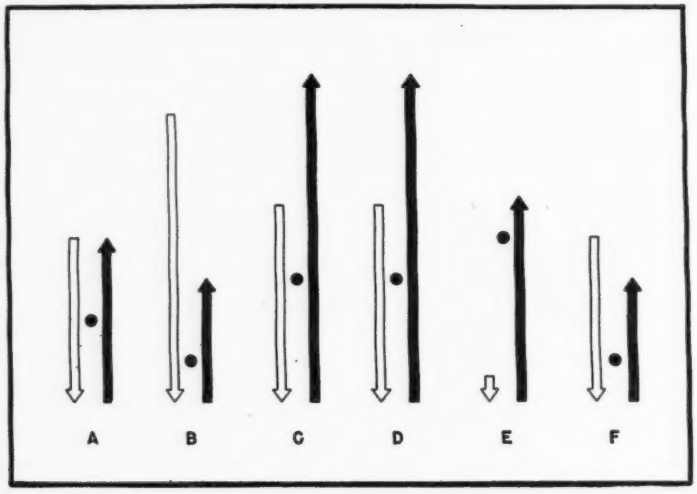

Fig. 2. SCHEMA SHOWING INFLUENCES WHICH AFFECT THE $\mathcal{Q}O_2$ of thyroid immersed in serum. The white arrows denote the depressing action of thyroid hormone, the black arrows the stimulating action of thyrotropic hormone. The black dots indicate the actual deviation of the $\mathcal{Q}O_2$. Schema *A* indicates the balance in the serum of normal persons, *B* that in classic Graves' disease, untreated, *C* that in acromegaly, *D* that in Graves' disease with dissociated ophthalmopathy, *E* that in untreated myxedema, and *F* that in treated myxedema.

In the case of classic Graves' disease we know from both the B.M.R. and blood iodine that the concentration of circulating thyroid hormone is increased. The white arrow is, therefore, lengthened as in figure 2, *B*. This excess undoubtedly is responsible for the lowering of the $\mathcal{Q}O_2$ of thyroid. Whether the black arrow should be changed in length is uncertain.

In the variety of Graves' disease with dissociation of ophthalmopathy and thyrotoxicosis, there is little or no thyrotoxicosis, judged by the B.M.R. and blood iodine levels. Therefore, we may draw the white arrow, figure 2, *C*, merely slightly longer than in the normal, figure 2, *A*. On the other hand the elevation which was found in this type in $\mathcal{Q}O_2$ indicates an excess of thyrotropic hormone, therefore the black arrow is lengthened. Such an interpretation also is in agreement with the findings of Hertz (4) on thyrotropic hormone excretion in the urine.

Interestingly enough the findings in acromegaly disclose an altogether similar situation, figure 2, *D*.

In untreated myxedema, thyroid hormone is obviously greatly reduced or absent from the circulating flood. The white arrow must, therefore, be made very short, figure 2, *E*. The marked elevation found in the $\mathcal{Q}O_2$ of thyroid indicates an excess of thyrotropic activity; therefore, the black arrow must be drawn much longer than the white. In passing it may be pointed out that the present findings on untreated

myxedema are quite in agreement with those of Hertz and Oastler (5) from this clinic in which it was shown "that blood serum and urine from myxedematous patients contain appreciable quantities of thyreotropic material."

In treated myxedema the results were variable. In 2 cases the $\mathcal{Q}O_2$ was depressed and in 2 slightly elevated. In schema *F* of figure 2, we have indicated that it is slightly depressed, although it is quite possible that in many such cases the balance shown in schema *A* might prevail.

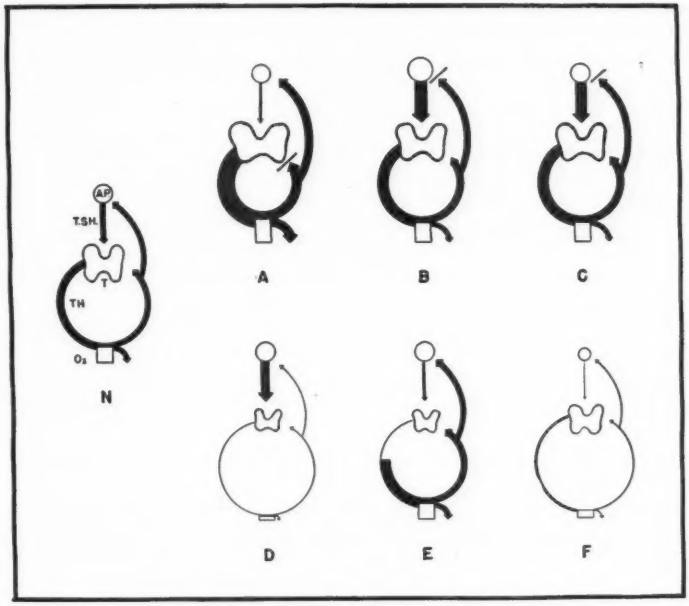

Fig. 3. SCHEMATA SHOWING THE HYPOTHETICAL HORMONIC RELATIONSHIPS BETWEEN THE PITUITARY AND THYROID GLANDS under normal conditions, schema *N*, and in classic Graves' disease, schema *A*, acromegaly, schema *B*, Graves' disease with dissociated ophthalmopathy, schema *C*, untreated myxedema, schema *D*, treated myxedema, schema *E*, and hypopituitarism, schema *F*.

Yet another attempt at diagrammatic interpretation is set forth in figure 3. Here in a series of schemata similar to those shown in a previous paper (1), it is indicated how the hormone, T.S.H., of the anterior lobe of the pituitary, A.P., acts upon the thyroid, T., to make its hormone, T.H., which maintains the rate of oxidation, O_2, of tissue cells. In schema *N* is shown the balance under normal circumstances. Some of the thyroid hormone is used in the tissues and eliminated. Some remains in circulation and acts on the thyroid directly and on the anterior pituitary in an inhibitory manner. In schema *A* is shown the situation in classic Graves' disease. It is inferred that the disturbance is primary in the thyroid and of such nature that the gland is incapable of inhibition by its own hormone. This is shown in the diagram by a barrier. This leads to an increased output of thyroid hormone by the thyroid, but the pituitary being normal is inhibited and the output of thyrotropic hormone is decreased.

In schemata *B* and *C*, figure 3, which represent the situation in acromegaly and in the form of Graves' disease with dissociated ophthalmopathy, it is suggested that the

A.P. puts out an excess of T.S.H. with some resulting increase in output of T.H. by the thyroid. The thyroid is also inhibited more strongly by this increase in its own hormone, with the result that its hyperfunction is slight or minimal. To account for the increased titer of T.S.H. in the blood of such patients which is actually found, a barrier is introduced indicating a hypothetical inability of T.H. to inhibit the A.P.

In schema *D* is represented the situation in untreated primary thyroid myxedema. The thyroid is incapable of making T.H. and therefore there is no inhibition of the A.P. and consequently an increased output of T.S.H. to which, however, the thyroid is incapable of responding. In schema *E* we have the same condition after treatment with thyroid.

In schema *F* is indicated the condition as we visualize it in primary pituitary hypofunction.

SUMMARY

The $\mathcal{Q}O_2$ of thyroid tissue of normal guinea pigs was determined by the method of Warburg. The media used were sera from normal persons as control, and from patients with various endocrine disorders as test material.

The serum of 5 acromegalics produced a rise in the $\mathcal{Q}O_2$ of guinea pig thyroid.

The serum of 4 patients with classic Graves' disease caused a depression of $\mathcal{Q}O_2$. That of one caused a 10% elevation. The serum of 2 of these patients retested after subtotal thyroidectomy caused very slight elevations in $\mathcal{Q}O_2$.

The serum of 4 patients (1 tested twice) of Graves' disease with dissociated ophthalmopathy caused elevation in $\mathcal{Q}O_2$ quite analogous to that in acromegaly.

In all of 4 cases of untreated primary myxedema there was an elevation of $\mathcal{Q}O_2$ and in treated myxedema there was slight elevation of $\mathcal{Q}O_2$ in 2 and reduction in 2 others.

In 2 cases of hypopituitarism the $\mathcal{Q}O_2$ was within the normal range of expected variation for the method.

The attempt has been made to explain these findings on the basis of a balance between circulating thyroid and thyrotropic hormone, such that an excess, relative or absolute, of the former will cause depression of $\mathcal{Q}O_2$ and that of the latter, elevation.

Thus in classic Graves' disease there is an excess, relative or absolute (the latter we may infer on the basis of elevated B.M.R.), of thyroid hormone activity. By thyroidectomy this is corrected. In acromegaly and in Graves' disease with dissociated ophthalmopathy there is an excess, relative or absolute, of thyrotropic activity. In untreated primary thyroid myxedema there is an absolute deficiency of thyroid hormone, and a relative or absolute excess of thyrotropic hormone. In treated myxedema the tendency is to the re-establishment of normal values. In hypopituitarism there is an absolute diminution in both thyrotropic and thyroid hormones.

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NOTES AND COMMENTS

THE ULTRACENTRIFUGAL SEDIMENTATION OF THE THYROTROPIC AND GONADOTROPIC PRINCIPLES OF CRUDE PITUITARY EXTRACTS*

In a previous publication from this laboratory[1] it was demonstrated that considerable concentration of the gonadotropic material in extracts of pregnant mare serum and of the urine of ovariectomized women is achieved by ultracentrifugation. In one experiment it was found that the thyrotropic activity of a crude extract of beef pituitary gland was not appreciably sedimented by centrifugation at 90,000 ×gravity† for 3 hours. Therefore, we have continued these experiments in order to determine whether or not the thyrotropic hormone can be sedimented and whether it can be effectively separated from the gonadotropic material by ultracentrifugal means. The data presented in this communication are derived from experiments designed to test these two points.

Two crude extracts of whole sheep pituitary were used. One extract (P9A), was prepared by extraction of acetone dried glands with dilute pyridine followed by partial fractionation according to Fevold.[2] The other preparation (P14C) was made by extraction of the dried glands with dilute NaOH, removal of the pH 4.2-insoluble material and precipitation of the supernatant fluid with flavianic acid.[3] Both preparations were rich in follicle-stimulating, luteinizing and thyrotropic activity.

Table 1

Prep.	Centrifugation			Volume of fractions			Concentration in bottom / Concentration in top		
	Time[1]	Centrifugal force[2]	Ft×10^{-8}[3]	Top	Middle	Bottom	Solids	Gonadotr.	Thyrotr.
	hr.	g.	dyne hr.	cc.	cc.	cc.			
P9AJ	3	100,000	2.94	2.0	1.6	1.4		2	2
P14CF	2.25	147,000	3.25	1.1	3.0	0.9	2.7	2 to 3	2 to 3
P9AK	5	91,000	4.48	1.2	2.5	1.2	2.2	4	2 to 3
P14CE	5	91,000	4.48	1.1	3.0	0.9	4	4	4
P14CH	6	156,000	9.19	1.2	2.5	1.3	5.8	8	8

[1] Coasting after shutting off of power is not included in these figures nor in calculations.
[2] Average field at center of tubes.
[3] $Ft = 4\pi^2 r n^2 t$ where r = mean radius of rotation; n = rev. per second; t = time in hours.

In the different experiments the speed and time of centrifugation were so adjusted that the product of the centrifugal force and its time of application varied over a wide range. After

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[1] Severinghaus, A. E., L. Levin and J. A. Chiles, Jr.: *Endocrinology* 23: 285. 1938.
† Average field at center of tubes.
[2] Fevold, H. L.: *Endocrinology* 24: 435. 1939.
[3] Smelser, G. K.: *Endocrinology* 23: 429. 1938.

each run the centrifuge was allowed to coast to a stop. To minimize stirring, a suction pipette with upturned tip was used for separating the contents of each tube into a top, middle and bottom fraction, corresponding fractions from the 10 tubes being pooled. The volume of each fraction was based on an approximation of the least amount of material required for dependable bio-assays, the intention being to separate the top and bottom fractions by as large a middle portion as possible. Total solids in each pooled fraction were determined by drying a small aliquot to constant weight.

In each case, the top and bottom fractions were quantitatively assayed for thyrotropic and gonadotropic activity. Thyrotropic activity was estimated by the chick thyroid method[3] and gonadotropic content by the ovarian weight response of immature rats or mice, groups of 6 to 10 animals being used for each of several dose levels. The dosages were so adjusted that the smallest produced little or no response. Thus an approximation of the amount of each fraction required to produce minimal effective stimulation was obtained. The degree of sedimentation was then expressed (table 1) as the ratio of the activity of the bottom fraction to that of the top.

The results obtained from 5 such experiments are presented in table 1. As is evident from the data, total solids, gonadotropic and thyrotropic activity are all sedimented at essentially equal rates and, as is to be expected, the amount of sedimentation is dependent on the product of the centrifugal force applied to the solution and the length of time of its application. At slow speeds applied for relatively short periods the bottom fraction was approximately twice as potent as the top with respect to thyrotropic as well as gonadotropic activity. Considerably greater differences in activity (as much as 8 times) between top and bottom fractions were obtained when higher speeds were applied for longer periods of time.

The fact that the total solids sedimented at a rate proportional to that of the physiological activity indicates that under the conditions of these experiments no purification was effected. This, however, does not exclude the possibility that by suitable alteration of the experimental conditions at least a partial separation of the inert proteins from the physiologically active components might be achieved. In this connection, it should be borne in mind that the preparations used in the present experiments were made from acetone-dried glands. It is possible that the material from fresh glands might behave differently.

The present results extend our earlier observations to include the gonadotropic principles of sheep pituitary gland as well as those of castrate urine and of pregnant mare serum in the ultracentrifugally sedimentable group of hormones. In addition it is demonstrated that the thyrotropic activity of pituitary extracts also sediments if sufficient centrifugal force is applied. The results are quite uniform in indicating that the 2 types of activity are sedimented at approximately the same rate. It must be emphasized, however, that this finding is not interpreted by us to mean that the same substance is responsible for gonadotropic and thyrotropic activity.

Summary. The gonadotropic and thyrotropic principles of crude sheep pituitary extract are sedimented by high speed ultracentrifugation.

The amount of sedimentation is proportional to the total centrifugal force applied, i.e., to the product of the speed and time of centrifugation.

The sedimentation rate of the gonadotropic and thyrotropic principles appears to be very similar and in proportion to the rate of sedimentation of the inert proteins.

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ASSOCIATION NOTICE

ANNOUNCEMENT OF THE ANNUAL MEETING

THE TWENTY-SIXTH ANNUAL MEETING of The Association for the Study of Internal Secretions will be held in Atlantic City, New Jersey on Monday and Tuesday, June 8th and 9th, 1942. The Hotel Traymore will be the headquarters for registration and for the scientific and business sessions. Room reservations should be made early with the Hotel Traymore.

The Chairman of the Local Committee is Dr. Matthew Molitch, 705 Pacific Avenue, Atlantic City, New Jersey.

PRESENTATION OF PAPERS

1. The title of the paper and a comprehensive abstract must reach the President, Dr. Edgar Allen, 333 Cedar Street, New Haven, Connecticut, not later than April 10, 1942.

Abstracts submitted should be in proper form for printing in the program. Not more than the first 200 words can be included in the printed abstract.

2. Non-members who wish to present papers must have their titles and abstracts introduced by members.

3. The abstracts of papers will be considered by the program committee and the final program announced about May 10th, 1942.

4. Papers will be limited to ten minutes for presentation. A ten-minute presentation is designed for condensed discussion of new investigations, either in laboratory or clinical phases of endocrinology. Previous publication or presentation before other societies of national membership may be cause for omitting a paper from the program.

5. Papers presented at the Annual Meeting may be submitted for publication to the Editor of ENDOCRINOLOGY or THE JOURNAL OF CLINICAL ENDOCRINOLOGY. Such submission is invited. Acceptance of a paper for the program does not necessarily mean its acceptance for publication.

You are invited to bring this announcement to the attention of your colleagues and associates who have endocrine research in progress.

NOMINATION OF OFFICERS

The By-Laws of the Association provide that nominations for all elective offices shall be made by a Nominating Committee and forwarded to the Secretary at least sixty days before the annual meeting. The Nominating Committee for the current year, appointed by the President and accepted by the Council, is as follows:

Dr. Earl T. Engle, *Chairman*, College of Physicians and Surgeons, 630 West 168th Street, New York City.

Dr. E. L. Sevringhaus, Wisconsin General Hospital, Madison, Wisconsin.

Dr. E. C. Kendall, 627 Eighth Avenue, S. W., Rochester, Minnesota.

The By-Laws also provide (Article V, Section 2) that "Any member of the Association may submit nominations to the Nominating Committee for its consideration." Nominations may be sent to the chairman, Dr. Earl T. Engle, before March 1st, 1942.

The terms of the following officers expire at the time of the annual meeting in Atlantic City in 1942.

President-Elect	E. Kost Shelton
Vice-President	E. M. K. Geiling
Secretary-Treasurer	Henry H. Turner

Council Members	*Publication Board*
David P. Barr	David P. Barr
John C. Burch	Oscar Riddle
E. C. Kendall	

THE E. R. SQUIBB & SONS AWARD

The E. R. Squibb & Sons award of $1,000.00 was established in 1939, and given first in 1940 to Dr. George W. Corner, and in 1941 to Dr. Philip E. Smith. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology. Nominations for the award in 1942 by members of the Association may be sent to the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, not later than March 1st. The nomination should be accompanied by a statement concerning the nominee's contributions to endocrinology and a bibliography of his most important publications.

Edgar Allen, *President*
Henry H. Turner, *Secretary*